

The inhibition of phosphodiesterase type 5 as a novel target for antidepressant action

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Abstract

Major depression is one of the most debilitating diseases of our time, while current antidepressant treatments remain deficient in several ways. The nitric oxide (NO) / cyclic guanosine monophosphate (cGMP) / cGMP-dependent protein kinase (PK-G) pathway shows promise as a novel target for the drug therapy of depression. A recent study from our laboratory reported an antidepressant-like response in the rat forced swim test (FST) following chronic (11 day) co-administration of the phosphodiesterase type 5 (PDE5) inhibitor sildenafil and the muscarinic acetylcholine (mACh) receptor antagonist atropine in Sprague Dawley rats. In the current study we explored the antidepressant-like properties of PDE5 inhibitors in Flinders Sensitive Line (FSL) rats, a genetic animal model of depression, and investigated the mechanism(s) that may be involved in the antidepressant-like activity of these drugs. We also evaluated possible anxiolytic-like activity following chronic PDE5 inhibition, examined the effects of sildenafil \pm atropine on frontal cortical and hippocampal mACh receptor densities and investigated the potential for sildenafil as a possible augmentation strategy to current antidepressant therapy. FSL rats were treated with vehicle/drug(s) for 14 days, whereafter immobility, swimming and climbing behaviours were measured in the FST, or time spent in social interaction in the social interaction test. Following decapitation, saturation binding studies were performed for the measurement of mACh receptor density. For the investigation of PK-G involvement, a subacute FST paradigm and Sprague Dawley rats were used. Chronic treatment of FSL rats with sildenafil or tadalafil (in combination with atropine) induced antidepressant- and anxiolytic-like responses in the FST and the social interaction test, respectively. The effects of known antidepressants were not potentiated by sildenafil in the FST. The dependency of the antidepressant-like response of sildenafil on the co-administration of atropine, as well as effects on behavioural correlates of serotonergic and noradrenergic neurotransmission were dose-related, suggesting that it may differentially affect the regulation of neurotransmission associated with antidepressant and depressogenic responses at different doses. Unlike the mood-regulating responses, however, the anxiolytic-like responses following chronic PDE5 inhibition does not appear to involve an interaction with the cholinergic system. We also demonstrated that the antidepressant-like mechanisms of sildenafil appear to involve cGMP-mediated activation of PK-G, but that unrelated mechanism(s) are also likely to play a role. Lastly, we demonstrated that the pro-cholinergic action of sildenafil does not involve up-regulation of frontal cortical and hippocampal mACh receptors. In summary, this project emphasises the potential of PDE5 inhibition as a novel antidepressant and anxiolytic strategy, and provides important insight into the specific neuronal mechanism(s) that may be involved in the antidepressant-like responses of inhibitors of this enzyme.

Abstrak

Major depressie is een van die mees ontmagtigende siektes van ons tyd, terwyl bestaande antidepressant-behandelings in verskeie opsigte oneffektief bly. Die stikstofmonoksied- (NO) / sikliese guanosienmonofosfaat- (cGMP) / cGMP-afhanklike proteïenkinase- (PK-G) weg toon belofte as nuwe teken vir die geneesmiddelbehandeling van depressie. Vanuit 'n onlangse studie in ons laboratorium is 'n antidepressant-agtige respons in die gedwonge swemtoets (GST) na chroniese (11 dae) ko-administrasie van die fosfodiësterase tipe 5 (PDE5) inhibeerder sildenafil en die muskariniese asetielholien (mACh) reseptor antagonist atropien in Sprague Dawley-rotte aangetoon. In die huidige studie het ons ondersoek ingestel na die antidepressant-agtige eienskappe van PDE5-inhibeerders in die Flinders Sensitive Line (FSL) rotte, 'n genetiese dieremodel van depressie, en na die meganisme(s) wat betrokke mag wees by die antidepressant-agtige aktiwiteit van hierdie geneesmiddels. Ons het verder moontlike ansiolitiese aktiwiteit na chroniese PDE5-inhibisie geëvalueer, die effekte van sildenafil ± atropien op die mACh reseptor digtheid in die frontale konteks en hippocampus ondersoek en ondersoek ingestel na die potensiaal van sildenafil as 'n versterkingstrategie tot bestaande antidepressant-terapie. FSL rotte is vir 14 dae met draer/geneesmiddel(s) behandel, waarna immobiliteit-, swem- en klimgedrag in die GST gemeet is, of tyd spandeer in sosiale interaksie in die sosiale interaksietoets. Na dekapitasie is saturasiebindingstudies uitgevoer vir die meting van mACh reseptor digtheid. Ten einde PK-G-betrokkenheid te ondersoek, is 'n subakute GST en Sprague Dawley-rotte gebruik. Chroniese behandeling van FSL-rotte met sildenafil of tadalafil (in kombinasie met atropien) het antidepressant- en ansiolitiese response in onderskeidelik die GST en die sosiale interaksietoets geïnduseer. Die effekte van bekende antidepressante is nie deur sildenafil in die GST potensieer nie. Die afhanklikheid van die antidepressant-agtige respons van sildenafil op die ko-administrasie van atropien, sowel as die effekte op gedragskorrelate van serotonergiese en noradrenergiese neurotransmissie was dosis-afhanklik, wat daarop dui dat dit die regulering van neurotransmissie geassosieer met antidepressant- en depressogene response by verskillende dosisse differensieel affekteer. Die ansiolitiese-agtige response na kroniese PDE5-inhibisie toon egter nie 'n interaksie met die cholinergiese sisteem nie. Ons het ook aangetoon dat die antidepressant-agtige meganisme van sildenafil oënskynlik cGMP-gemedieerde aktivering van PK-G behels, maar dat die pro-cholinergiese werking van sildenafil nie die opregulering van mACh reseptore in die frontale korteks of hippocampus behels nie. In samevatting beklemtoon hierdie projek die potensiaal van PDE5-inhibisie as 'n nuwe antidepressant- en ansiolitiese strategie, en verskaf dit belangrike insigte in die spesifieke neuronale meganisme(s) wat spesifiek betrokke mag wees by die antidepressant-agtige response van inhibeerders van hierdie ensiem.

Introduction

Chapter 1

This introductory chapter serves as an orientation to the thesis and study as a whole, and is therefore very concise. A more elaborate literature study is presented in Chapter 2.

1.1 Thesis layout

This Ph.D. thesis is compiled in the format of the article model of the North-West University. This implies that the main body of the thesis (methodology and experimental data) will be provided as three scientific manuscripts prepared for submission to be published in an international peer-review journal, with any additional data relating to the study as a whole being presented in addenda.

The *Introduction* chapter (Chapter 1) provides a general and concise orientation to the thesis and study, including the problem statement, primary study objectives and the study layout. A review of the relevant literature background of the study is provided in Chapter 2, and will be more comprehensive than that presented in the manuscripts. Chapters 3, 4 and 5 will contain the key findings of this project in the form of three scientific manuscripts, prepared for submission to a journal that will be indicated and in accordance with the house style of that particular journal, as laid down in the "Instructions to the Author". Two of these articles have been provisionally accepted for publication, and one paper is ready for submission. A final chapter (Chapter 6), summarises, discusses and concludes the study as a whole, incorporating all three manuscripts, as well as the data presented in the addendum. The *Addenda* contain additional data that were not incorporated in the manuscripts, as well as the relevant "Instructions to the Author" of the manuscripts prepared for publication as well as abstracts of congress contributions. The references for the articles are provide separately for each paper, whereas the bibliography for the sources referred to in the other chapters is presented at the end of the thesis.

1.2 Problem Statement

The World Health Organization (WHO) estimate that around 121 million people world-wide currently suffer from depression, making depression one of the most debilitating diseases in Africa and in the rest of the world. Furthermore, major depression is considered a serious and

debilitating psychiatric disorder which disrupts social functioning, causes severe suffering to the individual, and is a major cause of suicide (World Health Organization, 2009).

Although antidepressant drugs are a major therapeutic intervention, these agents have a delayed onset of action, display treatment resistance in roughly a third of patients, and also evoke unwanted, sometimes intolerable side-effects in many patients (Baldessarini *et al.*, 2002; Trivedi *et al.*, 2006). Despite major advances in our understanding of the biological basis of depression and antidepressant action, there has been very limited progress in the development of novel antidepressants since the introduction of the selective serotonin reuptake inhibitors (SSRIs) in the 1970's. Two exceptions may include the introduction of tianeptine and agomelatine, respectively claimed to be neuroprotective and to restore disrupted circadian rhythms as their primary mechanisms of antidepressant action. However, these drugs still have direct effects on monoaminergic neurotransmission (Mennini *et al.*, 1987; Millan *et al.*, 2003), so that their mechanisms of action remain partly or directly dependent on the classical monoaminergic hypothesis of depression. A limited number of experimental drugs and treatment strategies have begun to target other neurological mechanisms, such as the glutamatergic signal transduction pathways, or cytokine-mediated inflammatory processes (Sanacora *et al.*, 2008; Leonard & Myint, 2009). In order to address the above-mentioned shortcomings, there is a pressing need to develop new antidepressant drugs and strategies targeting novel neurobiological processes to more effectively treat depression.

One of the more recent hypotheses of the biological basis of depression, referred to as the neuroplasticity hypothesis, involves the glutamate / nitric oxide (NO) / cyclic guanosine monophosphate (cGMP) / cGMP-dependent protein kinase (PK-G) signal transduction pathway (Kleppisch & Feil, 2009). However, current preclinical studies describing the effects of modulating the glutamate/NO/cGMP/PK-G pathway on depressive-like behaviour in animal models are contradictory. In an attempt to elucidate the involvement of this pathway in mood-regulation, a recent study in our laboratory investigated the antidepressant-like properties of sildenafil in a rat model of depression (Brink *et al.*, 2008). This drug selectively inhibits phosphodiesterase type 5 (PDE5), the enzyme responsible for cGMP degradation, thereby enhancing cGMP signalling. Indeed, earlier studies have highlighted the possible involvement of cGMP signalling in the action of lithium, a well known mood stabilising agent (Harvey *et al.*, 1990a; Harvey *et al.*, 1990b). An important result from the study performed by Brink *et al.* (2008) was that the antidepressant-like activity of sildenafil was only revealed when co-administered with a muscarinic acetylcholine (mACh) receptor antagonist (atropine), and implied a novel cGMP-cholinergic interaction in mood-regulation. These novel and interesting results now need to be confirmed in additional animal models of depression, while further experimentation is required to elucidate the antidepressant-like properties of other PDE5

inhibitors, and also to learn more of the mechanism(s) involved in their antidepressant-like action.

1.3 Study Objectives

The primary objective of the current study was to investigate the antidepressant-like properties of the PDE5 inhibitors, sildenafil and tadalafil, in a genetic rodent model of depression, and to obtain more insight into the mechanisms involved in their antidepressant-like action.

More specifically, the study aimed to:

- confirm the antidepressant-like activity of sildenafil + atropine in a genetic rat model of depression;
- explore the antidepressant-like properties of sildenafil by investigating (1) the dose-response relationship of the antidepressant-like properties of sildenafil, (2) the role of the cGMP-cholinergic interaction in this regard and (3) to investigate the behavioural correlates of monoaminergic neurotransmission in this response;
- evaluate antidepressant-like activity of a structurally distinct PDE5 selective inhibitor (tadalafil) and the dependency of any such activity on mACh receptor antagonism;
- investigate the involvement of PK-G in the antidepressant-like action of sildenafil;
- explore possible augmentation of known antidepressants with sildenafil in terms of potentiation of efficacy, as well as a possible hastening of their onset of action;
- evaluate possible anxiolytic effects of PDE5 inhibitors with or without mACh receptor blockade; and
- to investigate whether the pro-cholinergic effect of chronic treatment with sildenafil is related to changes in cortical and hippocampal mACh receptor densities.

Confirm the antidepressant-like properties of sildenafil

Firstly, we evaluated whether the previously reported antidepressant-like action of sildenafil (Brink *et al.*, 2008) can be replicated in a pathological rat model of depression. To this end, we used a genetic model in which rats are inherently more susceptible to depression-like behaviour. Flinders Sensitive Line (FSL) rats present with specific depression-like characteristics compared to their corresponding controls, the Flinders Resistant Line (FRL) rat (Overstreet *et al.*, 2005a). The use of FSL rats offers several advantages. Firstly, this model exhibits improved aetiological validity, since a genetic predisposition to depression has also been implicated in the aetiology of depression in humans (Sullivan *et al.*, 2000; Rice *et al.*, 2002). Secondly, this rodent model more closely resembles the clinical situation in humans by demonstrating reversal of the depression-like behavioural deficits following chronic, but not

acute antidepressant treatment (Overstreet, 1993; Overstreet *et al.*, 1995a; Dremencov *et al.*, 2004). Immobility behaviour, as measured in the forced swim test (FST), was used as a parameter of the depression-like state of the rats, whereas a distinction between the active behaviours in this test (i.e. swimming and climbing) provide an indication of specific monoamine transmission pathways involved (i.e. serotonergic and noradrenergic, respectively), as has been proposed by Cryan *et al.*, (2002). The study investigating these objectives is presented in Chapter 3.

Investigate the dose-dependency of the antidepressant-like properties of sildenafil

Given previous evidence for dualistic and dose-dependent actions for NO donors and NOS inhibitors in rodent models of depression (da Silva *et al.*, 2000; Inan *et al.*, 2004), and our hypothesis of the activation of both pro- and antidepressive mechanisms by sildenafil, we explored whether the activation of these mechanisms may be dose-dependent. Specifically, we investigated the dependency of the sildenafil-cholinergic interaction, as well as the effects on distinct active behaviours in this test (i.e. swimming and climbing) on the dose used for sildenafil. The study investigating these objectives is presented in Chapter 3.

Evaluate the antidepressant-like action of a structurally distinct PDE5 inhibitor (tadalafil)

It is currently unclear whether the antidepressant-like action of sildenafil is a property unique to this drug, and whether this property is shared by other PDE5 inhibitors. Therefore, the possible antidepressant-like properties of tadalafil, a structurally unrelated PDE5 inhibitor, was evaluated in FSL rats and the pattern of response in the FST compared to that of sildenafil. The study investigating these objectives is presented in Chapter 3.

Determine PK-G involvement in the antidepressant-like action of sildenafil

In order to confirm a select involvement of the cGMP signalling cascade in the antidepressant-like response of sildenafil, we determined whether the antidepressant-like action of sildenafil + atropine (Brink *et al.*, 2008) is directly dependent on PK-G activation, secondary to the inhibition of PDE5 which would lead to an increase in the concentration of cGMP. We firstly evaluated whether direct activation of PK-G using a selective PK-G activator (with or without concurrent mACh receptor blockade) can produce an antidepressant-like response in the FST. Secondly, we studied the converse, namely whether inhibition of PK-G with a selective PK-G inhibitor, can abolish the antidepressant-like response of sildenafil + atropine. The PK-G modulators that were used in this objective require intracerebroventricular (i.c.v.) administration. Given the considerable risk for infection during chronic i.c.v. treatment regimes, we opted to use normal Sprague-Dawley rats and the acute FST paradigm for these experiments. The study investigating these objectives is presented in Chapter 4.

Exploring the interactions of sildenafil with other antidepressants

Here we investigated whether sildenafil may be a useful adjunct to current antidepressants in the alleviation of depression. Possible augmentation of the efficacy, as well as a potential hastening of the onset of the antidepressant-like responses, was explored in FSL rats in the FST with fluoxetine in combination with sildenafil + atropine, or with sildenafil in combination with imipramine, an antidepressant with inherent antimuscarinic properties. The study investigating these objectives is presented in Addendum A.

Investigate possible anxiolytic effects for PDE5 inhibitors

A considerable comorbidity between depression and anxiety disorders has been described (Kessler *et al.*, 1994; Zimmerman *et al.*, 2000; Kessler *et al.*, 2005), and known antidepressants are also used clinically in the treatment of anxiety. Since the NO/cGMP/PK-G pathway has been shown to play a role in the regulation of anxiety (Eroglu & Caglayan, 1997; Volke *et al.*, 1997; Volke *et al.*, 2003a; Volke *et al.*, 2003b; Gilhotra & Dhingra, 2009), we investigated whether PDE5 inhibitors, alone or in combination with a mACh receptor antagonist, may have anxiolytic properties. Since FSL rats present with increased anxiety-like behaviour in the social interaction test relative to FRL control rats (Overstreet *et al.*, 2004), this genetic model is appropriate for the assessment of possible anxiolytic-like properties of drugs. The study investigating these objectives is presented in Chapter 5.

Investigate effects of sildenafil on mACh receptor density

Sildenafil has been shown to have pro-cholinergic properties (Devan *et al.*, 2004; Patil *et al.*, 2004a; Brink *et al.*, 2008), an effect that is believed to be depressogenic (Janowsky *et al.*, 1972; Janowsky *et al.*, 1994) and to be involved in the cGMP-cholinergic interaction reported for sildenafil in the FST (Brink *et al.*, 2008). Given the apparent role for mACh receptor antagonism in the antidepressant-like response of sildenafil, we investigated whether sildenafil may alter mACh receptor density in brain areas associated with depression (i.e. hippocampus and frontal cortex). The study investigating these objectives is presented in Addendum A.

1.4 Study Layout

This project was carried out in three separate experimental phases. The strain of rats used, treatment duration, study objectives, behavioural and molecular tests employed and the laboratory in which the experiments were carried out, are provided in Table 1.1.

Table 1.1 Experimental layout

	Phase 1	Phase 2	Phase 3
Animals	FSL and FRL rats	Sprague-Dawley rats	FSL and FRL rats
Treatment	Chronic 14 days	Subacute 2 days	Chronic 14 days
Objectives	<ol style="list-style-type: none"> 1) Confirm antidepressant-like activity for sildenafil + atropine in a genetic model 2) Evaluate dose-response relationships 3) Investigate PDE5 involvement 4) Explore augmentation strategies with known antidepressants 5) Measure mACh receptor densities 	<ol style="list-style-type: none"> 1) Investigate the involvement PK-G in the antidepressant-like properties of sildenafil 	<ol style="list-style-type: none"> 1) Evaluate the effect of chronic PDE5 inhibition on anxiety-like behaviour
Experiments & Assays	<ul style="list-style-type: none"> • Forced swim test (FST) • Open field test • Saturation binding assays 	<ul style="list-style-type: none"> • Forced swim test (FST) • Open field test 	<ul style="list-style-type: none"> • Social interaction test
Laboratory	Unit for Drug Research and Development North-West University Potchefstroom South Africa	Centre for Psychiatric Research Århus University Hospital Risskov Denmark	Unit for Drug Research and Development North-West University Potchefstroom South Africa

Literature Background

Chapter 2

In this chapter, a broader and more in-depth discussion of the relevant literature will be presented than that provided in the manuscripts. Firstly, major depression will be discussed by means of a description of some general aspects of the disease (relevant statistics, criteria for diagnosis, and the aetiology and brain anatomy involved) as well as a discussion of the current hypotheses that describe the neurobiological basis for depression. Secondly, the role of the glutamate/NO/cGMP/PK-G pathway in depression will be described in the context of the neurobiology associated with mood-regulation. Thirdly, the role of phosphodiesterase type 5 (PDE5) in the brain, and psychotropic effects of PDE5 inhibitors, with specific reference to their potential use in the treatment of depression, will be discussed. A brief overview of different animal models of depression and for antidepressant drug testing will also be provided, including a discussion of some of the advantages and limitations of these models.

2.1 Major depression

2.1.1 Statistics

Mood disorders are amongst the most prevalent forms of mental illnesses. A study in the U.S.A. has reported that 1 out of 6 people will develop clinical depression at sometime during their lifetime (Kessler *et al.*, 2005). In addition, it is projected that by 2020 depression will be the 2nd most debilitating disease across all age groups, whereas it is already the 2nd most debilitating illness for the age group 15-44 years old (World Health Organization, 2009). Among the population of South Africa, a lifetime prevalence of 9.7% has been reported for depression (Tomlinson *et al.*, 2009). In addition, the prevalence of HIV-AIDS in this country further contributes to the incidence of depression (Owe-Larsson *et al.*, 2009). This is triggered by the emotional trauma and stigmatisation that HIV-positive patients commonly experience, and is further complicated by the occurrence of anti-retroviral side-effects and the neurocognitive complications associated with HIV-AIDS and its treatment (Owe-Larsson *et al.*, 2009).

Depression is a serious disorder that is associated with a great degree of suffering, as well as being a major cause for suicide (i.e. relatively high mortality), with a reported 1 million lives being claimed worldwide by suicide each year (Goldsmith *et al.*, 2002). In addition, depressed patients are also more likely to develop cardiovascular disease (Halaris, 2009) and type 2 diabetes (Knol *et al.*, 2006), whereas depression complicates the prognosis of several other chronic illnesses (Evans *et al.*, 2005; Gildengers *et al.*, 2008).

2.1.2 Diagnosis of major depression

According to the Diagnostic and Statistical Manual of Mental Disorders (4th ed) (American Psychiatric Association, 1994), an episode of major depression is diagnosed as a period of 2 weeks or longer in which a patient presents with 5 or more of the symptoms listed in Table 2.1, and when these symptoms disrupt the normal social and/or occupational functioning of the patient. The presence of at least one of the first two symptoms in this list is a prerequisite for a diagnosis of major depression to be made.

Table 2.1 Diagnostic criteria for the diagnosis of major depression

- Depressed **mood**
- Diminished **interest** or **pleasure** in most activities
- Significant **weight loss** or gain, or decrease or increase in **appetite**
- **Insomnia** or hypersomnia
- **Psychomotor** agitation or retardation
- Fatigue or loss of energy
- Feelings of **worthlessness**, or excessive or inappropriate **guilt**
- Diminished ability to **think** or **concentrate**, or **indecisiveness**
- Recurrent thoughts of death or **suicidal** ideation

It is clear from the criteria in Table 2.1 that the diagnosis of major depression, as opposed to most other illnesses (such as cardiovascular disease or cancer) is not based on objective diagnostic tests, but rather on a variable set of relatively subjective symptoms. Therefore, rather than being viewed as a single disease, depression is characterised as a heterogeneous syndrome consisting of numerous distinct symptoms.

2.1.3 Aetiology of depression

Epidemiologic studies have suggested that 40 to 50% of the risk for developing depression is genetic (Sanders *et al.*, 1999; Fava & Kendler, 2000). This would imply that depression is a highly heritable disorder, although there is still only speculation as to the specific genes that could be involved. Given the fact that depression is a complex phenomenon with many genes potentially involved in its aetiology (Burmeister, 1999), the undertaking of identifying these genes is particularly challenging. Furthermore, the aetiology of depression is only partly genetic, with non-genetic factors also playing an important role. These include chronic emotional stress and can be as diverse as viral infections (e.g., Borna virus) or interferon alpha (IFN- α) therapy (Akiskal, 2000; Wichers *et al.*, 2005), that act as environmental triggers for the development of depression in genetically susceptible individuals.

The role of stress appears to be of particular importance in the aetiology of depression. Indeed, this disease is often described as a stress-related disorder, and there is good evidence that episodes of depression often occur in the presence of some form of prior or ongoing stress (Kendler *et al.*, 1999; Hammen *et al.*, 2009). However, stress per se is not sufficient to cause depression, since not all people that are exposed to a stressful event go on to develop depression, while those who do develop depression usually do so following only a mild stressor (Nestler *et al.*, 2002). In addition, severe traumatic stress (such as that experienced during war or rape) typically does not induce depression, but instead may cause post-traumatic stress disorder (PTSD) (Nestler *et al.*, 2002), a mental disorder that is clinically distinct from depression, although there is often a significant comorbidity between depression and PTSD (Neria & Bromet, 2000). These observations suggest that the aetiology of depression may involve interactions between a genetic predisposition to depression as well as various environmental factors, rendering the mechanisms of such interactions an important focus of investigation.

2.1.4 The neuroanatomy of depression

Although many brain regions have been implicated in depression, there is still no consensus on the particular neuronal circuitry(ies) that are involved in the regulation of mood (Nestler *et al.*, 2002; Krishnan & Nestler, 2008). This is in striking contrast to neuropsychiatric disorders such as Parkinson's disease, Huntington's disease, and Alzheimer's disease, for which pathological lesions in specific brain regions, involving well-defined neural circuitries, have been identified.

It is highly probable that many brain regions mediate the diverse symptoms of depression. This is supported by human brain imaging studies, which have demonstrated changes in blood flow or other measures in several brain regions, including regions of the prefrontal and cingulate cortex, hippocampus, striatum, amygdala, and thalamus (Drevets, 2001; Liotti & Mayberg, 2001). In addition, anatomical studies of the brains of depressed patients, as obtained with autopsy, have also found abnormalities in many of these brain regions (Rajkowska, 2000; Manji *et al.*, 2001; Sheline, 2003). However, some of the imaging and autopsy studies have yielded contradictory findings, and the role of these regions in depression remains uncertain (Krishnan & Nestler, 2008).

Knowledge of the normal neuropsychological and other mental functions of the respective brain regions implicated in depression may suggest the depressive symptoms to which they contribute. For example, the neocortex and hippocampus may mediate the cognitive aspects of depression, such as the impairment of memory and feelings of worthlessness, guilt and suicidal thoughts. The striatum (particularly the ventral striatum or nucleus accumbens (NAc)) and amygdala are involved in emotional memory, and could therefore mediate anhedonia (decreased drive and reward for pleasurable activities), as well as anxiety, that is also present in

many depressed patients (Hasler *et al.*, 2004; Ressler & Mayberg, 2007). It has been suggested that dysfunction of the hypothalamus may be involved in the neurovegetative symptoms of depression, including excessive or impaired sleep, appetite or energy, as well as a loss of sex-drive (Nestler *et al.*, 2002). These various brain regions operate in a highly interactive manner, and may represent, at least in part, the neural circuitry involved in neurobiology of depression.

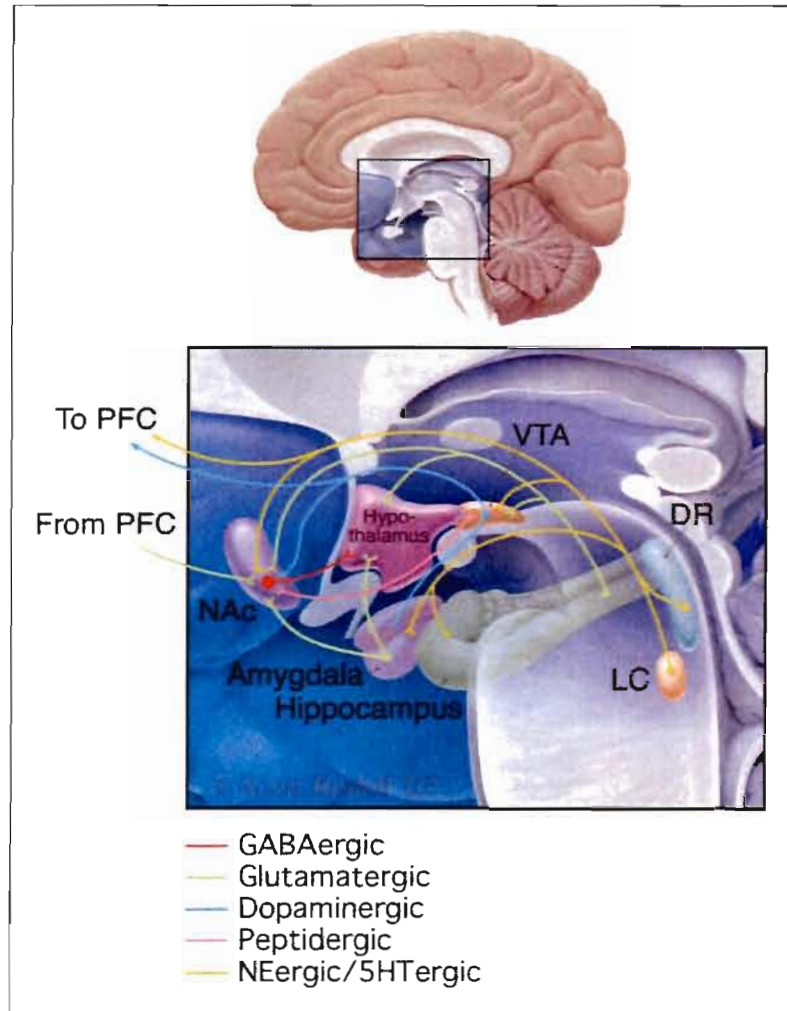


Figure 2.1 The neural circuitry of depression. The figure shows only a few of the many known interconnections among these brain regions. The ventral tegmental area (VTA) provides dopaminergic input to the NAc, amygdala, PFC, and other limbic structures. Noradrenergic (from the locus coeruleus, LC) and serotonergic (from the dorsal raphe, DR) neurons innervate all of the areas in this illustration. There are also strong connections between the hypothalamus and the VTA-NAc pathway (Nestler *et al.*, 2002).

Figure 2.1 is a simplified illustration of several neural circuits that have been implicated in the pathology of depression. The hippocampus and prefrontal cortex (PFC) areas have been the

focus of a large amount of research in this regard. However, there is an increasing realisation that several sub-cortical structures that are implicated in reward, fear, and motivation may also be involved (Yadid *et al.*, 2001). These include the nucleus accumbens (NAc), amygdala, and hypothalamic regions.

2.1.5 Neurobiological hypotheses of depression

Several hypotheses have been formulated to explain the neurobiological basis of depression and also to identify novel targets for antidepressant strategies. These focus on different components of the disorder and are based on different observations, but are not mutually exclusive. Rather, they overlap in many instances, and some hypotheses, particularly the more recent ones, can be viewed as unifying theories that can simultaneously explain observations that could not be explained by previous hypotheses.

2.1.5.1 The monoamine hypothesis

The modern history of antidepressant drug therapy started in the early 1950s when isoniazid and iproniazid, drugs that were initially developed for the treatment of tuberculosis, were found to have mood elevating effects in patients with tuberculosis and depression (Selikoff & Robitzek, 1952; Salzer & Lurie, 1953). Subsequently, it was found that iproniazid was capable of inhibiting monoamine oxidase (MAO) (Griesemer *et al.*, 1953). A few years later, the antidepressant efficacy of imipramine, a tricyclic compound with structural resemblance to the antipsychotic drug chlorpromazine, was discovered (Kuhn, 1958). Some of these facts lead to the postulation of the classical monoamine theory of depression in the 1960's (Schildkraut, 1965), which stated that depression is caused by a deficiency in monoaminergic activity in the brain, and that depression can be alleviated by drugs that increase monoaminergic neurotransmission.

The search for compounds that were structurally related to imipramine has yielded several tricyclic antidepressants that are still in clinical use today. Mianserin was the first "atypical" antidepressant that did not inhibit the reuptake of monoamines or MOA (Leonard, 1978). Instead, this drug was found to act by enhancing noradrenergic neurotransmission by blocking presynaptic α_2 -adrenergic autoreceptors. In a continued search for novel antidepressants, the selective serotonin reuptake inhibitors (SSRIs), e.g. fluoxetine and paroxetine, were introduced in the late 1980's and early 1990s (Fuller, 1995), and during the same period, mirtazapine, a drug that antagonises α_2 -adrenergic autoreceptors as well as serotonergic 5-HT₂ and 5-HT₃ receptors, was discovered (Smith *et al.*, 1990). All of these drugs modulate monoaminergic neurotransmission, thereby still supporting the monoaminergic hypothesis of depression.

The monoaminergic hypothesis of depression suffered several drawbacks by failing to explain a number of observations. For instance, changes in synaptic monoamine concentrations occur essentially immediately following the administration of antidepressants, whereas the therapeutic antidepressant response requires the continuous administration for several weeks with these drugs (Baldessarini, 1989). In addition, other drugs that also increase brain monoaminergic activity (e.g. cocaine and amphetamine) are not clinically effective antidepressants (Fischman & Foltin, 1991). More recent thinking regarding the pathophysiology of depression and antidepressant action suggest that drugs that acutely increase monoamine concentrations ultimately activate secondary effects on molecular and cellular plasticity (Nestler *et al.*, 2002; Ansorge *et al.*, 2007). These latter changes eventually facilitate the restoration of synaptic connectivity needed for normal neurotransmission to take place, and thereby alleviate depression (Manji *et al.*, 2003). Furthermore, these effects occur over a prolonged period of treatment, and appear to rely on alterations in gene expression. For example, it has been suggested that chronic SSRI treatment ultimately leads to the upregulation of the transcription factor, CREB (cAMP response element binding protein), an effect that correlates directly with the onset of antidepressant-like effects in animal models (Pittenger & Duman, 2008).

An interesting development in the search for novel antidepressants was the discovery of tianeptine, a drug that enhances the reuptake of serotonin in the synaptic cleft (Mennini *et al.*, 1987). This synaptic action is in contrast to the mechanisms of conventional antidepressants, such as SSRIs and tricyclic antidepressants, which inhibit the reuptake of monoamines. Despite this apparent paradox, tianeptine has been shown to exhibit antidepressant-like activity in animals (Broqua *et al.*, 1992; Rogoz *et al.*, 2008) as well as clinical efficacy equal to standard antidepressant regimes (Guelfi, 1992; Wagstaff *et al.*, 2001). Recent studies investigating the antidepressant properties of tianeptine have focussed on its putative role in neuroplasticity (Uzabay, 2008), a hypothesis of depression that will be discussed in more detail below (see § 2.1.5.5). Taken together, these observations prompted a re-evaluation of the biochemical basis of depression, and suggested that the clinical syndrome of depression cannot be fully explained by the monoaminergic hypothesis alone. Therefore, a unifying hypothesis that incorporates and explains a wide spectrum of observations is needed.

2.1.5.2 Cholinergic hypothesis

The cholinergic hypothesis of depression evolved out of the cholinergic-adrenergic imbalance theory described in the early 1970's, which suggested that an overactivity of cholinergic- over noradrenergic neurotransmission causes depression, whereas the opposite imbalance leads to mania (Janowsky *et al.*, 1972). Later, it was proposed that depressed individuals exhibit a cholinergic supersensitivity, which could be observed as an exaggerated behavioural or hormonal response to cholinergic agonists (Janowsky *et al.*, 1994). In addition, it has been

suggested that muscarinic receptors in the nucleus accumbens may be involved in depression and antidepressant action (Chau *et al.*, 2001), whereas another study has reported that the antimuscarinic drug, scopolamine, exerts an antidepressant effect as an augmentation strategy with other antidepressants in treatment-resistant patients (Furey & Drevets, 2006). Unfortunately, the cholinergic hypothesis of depression has received relatively little attention, probably due to the fact that anticholinergic drugs failed to materialise as effective antidepressants.

A recent study from our laboratory (Brink *et al.*, 2008) demonstrated a novel interaction between the cholinergic system and the nitric oxide (NO) / cyclic guanosine monophosphate (cGMP) / cGMP-dependent protein kinase (PK-G) pathway that shows promise as a novel target for antidepressant action. Furthermore, the now fashionable (but maybe enlightened) approach of integrating the distinct hypotheses of depression into a single, multifaceted hypothesis, may see the return of the cholinergic theory to the forefront of antidepressant research.

2.1.5.3 HPA-axis hyperactivity hypothesis

Hypothalamic-pituitary-adrenal-axis (HPA-axis) hyperactivity and defective HPA-axis glucocorticoid feedback mechanisms are widely reported neurobiological alterations in major depression (Pace *et al.*, 2007). In addition, patients with major depression have been shown to exhibit increased concentrations of cortisol in plasma, urine, and cerebrospinal fluid (Pariante & Miller, 2001). Depressed patients have also been shown to exhibit an exaggerated cortisol response to adrenocorticotropin hormone (ACTH) (Nemeroff, 1996).

The exaggerated HPA-axis activity observed in patients with major depression is believed to largely result from hypersecretion of corticotropin-releasing factor (CRF) (Pariante & Miller, 2001). There is evidence that this may contribute to the behavioural features of major depression, given that administration of CRF has been shown to induce behavioural changes in animals that are comparable to those seen in human depression (e.g. alterations in mood, appetite, sleep, locomotor activity and cognition) (Nemeroff, 1996). The mechanism underlying increased CRF release during depression is believed to be related to the failure in the negative feedback regulation of cortisol to suppress CRF secretion, and is suggested to be a consequence of glucocorticoid resistance involving glucocorticoid receptors (Plotsky *et al.*, 1998; Pariante, 2004). Disturbances in the HPA-axis is also regarded as a general feature of disturbed circadian rhythms evident in depression, and a motivation for the development of new antidepressants, such as agomelatine, that specifically target the bio-rhythms in the suprachiasmatic nucleus of the hypothalamus (Monteleone & Maj, 2008). Elevated cortisol levels for sustained periods of time may ultimately lead to damage of hippocampal neurons, a region that is implicated in mediating the symptoms of depression (McEwen, 2000; Sapolsky, 2000). Indeed, brain imaging studies have demonstrated a reduction in the volume of the

hippocampus of depressed subjects (Sheline, 2003; Duman, 2004). However, in addition to glucocorticoid-induced neurotoxicity, the neuronal atrophy seen in depression is also thought to involve defective neuroplastic mechanisms (Duman & Monteggia, 2006; Schmidt & Duman, 2007), and will be described in more detail in § 2.1.5.5.

2.1.5.4 Immunological hypothesis

Major depression is also associated with immune activation. Increased plasma and cerebrospinal fluid concentrations of a variety of cytokines and their receptors, including IL-1, IL-2, IL-6 and TNF- α have been reported in depressed patients, and these immune abnormalities are restored following antidepressant treatment (Raison *et al.*, 2006). In addition, the abovementioned cytokines have been shown to lead to "sickness behaviour" in animals and humans, a condition that shares a number of symptoms with major depression, including alterations in mood, neurovegetative function and cognition (Dantzer, 2004). Indeed, patients treated with IFN- α for cancer or viral infections such as hepatitis C often present with several of the diagnostic criteria of major depression (Renault & Hoofnagle, 1989; Muraoka *et al.*, 1996). The specific mechanism(s) by which cytokines affect behaviour are believed to be related to their effects on both neurotransmitter function and synaptic plasticity (Raison *et al.*, 2006). In addition, the effects of cytokines on the neuroendocrine system during depression is well described, and may be related in part to their effects on the signalling pathways of glucocorticoid receptors, that have been suggested to be involved in glucocorticoid resistance (Pace *et al.*, 2007). Therefore, it appears that the immune system and neuroendocrine systems are strongly interconnected in the neuropathology of depression, and the relationship between these systems in the regulation of affective behaviour is a focus of ongoing research.

2.1.5.5 Neuroplasticity hypothesis

As mentioned in § 2.1.5.3, volumetric decreases have been observed in the hippocampus and other forebrain regions of patients suffering from long-term depression (Sheline, 2003; Duman, 2004). This has led to a popular (and more unifying) hypothesis of depression that implicates decreases in neurotrophic factors that regulate plasticity within the adult brain (Manji *et al.*, 2003; Duman & Monteggia, 2006), whilst still accounting for altered monoaminergic neurotransmission and other changes in neurotransmission. According to this hypothesis, drugs that acutely increase monoamine concentrations will ultimately activate secondary effects on molecular and cellular plasticity (Nestler *et al.*, 2002; Ansorge *et al.*, 2007). These changes may facilitate the restoration of synaptic connectivity needed for normal neurotransmission to take place, and thereby relieve depression (Manji *et al.*, 2003). Furthermore, the glutamate/NO/cGMP/PK-G signalling pathway is also believed to play a key role in neuroplasticity (Zarate *et al.*, 2003; Calabrese *et al.*, 2007; Kleppisch & Feil, 2009), and drugs

that modulate this pathway have been shown to exert antidepressant-like activity (Zarate *et al.*, 2002b; Zarate *et al.*, 2003). As this pathway is of particular significance to the current study, its role in depression and antidepressant action will be described in more detail (compared to the abovementioned theories of depression).

Several key observations have suggested a role for neuroplasticity in depression (described below). These include results that have shown that stress and depression reduce neurotrophin expression, antidepressants increase neurotrophin expression and administration of neurotrophins produces antidepressant-like effects in animals. In addition, several studies have demonstrated that adult neurogenesis may be involved in the neurobiology of depression and in the mechanism of action of antidepressants.

Stress and depression reduce neurotrophin expression

The majority of studies investigating the role of neurotrophins in depression have focused on the role of brain-derived neurotrophic factor (BDNF). Support for the role of BDNF in depression has come from a large number of preclinical studies, demonstrating that several forms of acute and chronic stress leads to a reduction of BDNF expression in the hippocampus, whereas chronic antidepressant treatment restores BDNF to control levels (Duman & Monteggia, 2006). The down-regulation of BDNF expression have been attributed to the effects of glucocorticoids (Barbany & Persson, 1992; Schaaf *et al.*, 1998), pro-inflammatory cytokines (Barrientos *et al.*, 2003), as well as a reduced stimulation of 5-HT_{2A} receptors (Vaidya *et al.*, 1997). In addition to BDNF, there is also evidence that the detrimental effect of stress may be mediated by a decreased expression of other types of neurotrophic and growth factors, such as nerve growth factor (NGF) and neurotrophin-3 (NT-3) (Ueyama *et al.*, 1997). Furthermore, the expression of another class of growth factors, vascular endothelial growth factor (VEGF) as well as the type 2 VEGF receptor, has been reported to be decreased following unpredictable stress in animals (Heine *et al.*, 2005). As VEGF has been shown to increase neurogenesis (see below) in the hippocampus (Palmer *et al.*, 2000), decreased expression of VEGF may also play a role in the neuroplastic deficiencies that are associated with depression. In further support of the neuroplasticity hypothesis is that the expression of BDNF is reduced in the hippocampus of depressed suicide victims, and increased in victims receiving antidepressant treatment at the time of death (Chen *et al.*, 2001; Karege *et al.*, 2005).

Antidepressants increase the expression of neurotrophins

Several classes of antidepressants significantly increase the expression of BDNF in the hippocampus, including SSRIs, serotonin-norepinephrine reuptake inhibitors (SNRIs), monoamine oxidase inhibitors (MAOIs), atypical antidepressants, as well as electroconvulsive seizures (ECS) (Nibuya *et al.*, 1995). The hippocampal expression of VEGF is also increased by antidepressant drugs (Warner-Schmidt & Duman, 2007) as well as electroconvulsive shock

(ECS) therapy (Newton *et al.*, 2003). Of note is that the upregulation of BDNF is dependent on chronic antidepressant treatment (Duman & Monteggia, 2006; Warner-Schmidt & Duman, 2007), and is therefore consistent with the time course for the onset of therapeutic action of antidepressants.

BDNF administration produces antidepressant-like effects in animals

Local infusions of BDNF into specific brain regions have been shown to evoke antidepressant-like effects in behavioural models of depression. Infusion of BDNF into the midbrain, hippocampus or lateral ventricles evokes antidepressant-like effects in the forced swim test (FST) (Siuciak *et al.*, 1997; Shirayama *et al.*, 2002; Hoshaw *et al.*, 2005), and the learned helplessness test (Siuciak *et al.*, 1997; Shirayama *et al.*, 2002). This said, it has become evident that increased BDNF levels in different brain areas may produce distinct effects on depression-like behaviour (Duman & Monteggia, 2006), and underlines the complexity of the neurobiological interactions that may regulate neuroplasticity in the brain, and subsequently influence behaviour.

Neurogenesis and depression

The abovementioned neurotrophic factors play an important role in adult neurogenesis, the process by which neural progenitors of the hippocampal subgranular zone (SGZ) divide to form new neurons that differentiate and integrate into the dentate gyrus (DG) of the hippocampus. In addition, it has been demonstrated that several antidepressants induce hippocampal neurogenesis and that inhibition of neurogenesis prevents the antidepressant-like action of most antidepressant treatments (Sahay & Hen, 2007; Pittenger & Duman, 2008). It is believed that antidepressant therapy, possibly via the regulation of CREB, increases the levels of several neurotrophic factors in the hippocampus that affects neurogenesis, including BDNF and VEGF (Nibuya *et al.*, 1995; Warner-Schmidt & Duman, 2007). However, the mechanism(s) by which the formation of new neurons may alleviate depression are not well understood. It is believed that intact neurogenesis may underlie the ability of the brain to adapt to new experiences, versus a maladaptive learning response that may lead to depression when neurogenesis is compromised (Krishnan & Nestler, 2008). However, it appears that decreased neurogenesis by itself does not cause depression, since depression-like behaviour is not induced by inhibition of neurogenesis in rodents (Santarelli *et al.*, 2003; Surget *et al.*, 2008), although intact neurogenesis is required for the antidepressant-like effects of several (but not all) antidepressants to be expressed (Zhao *et al.*, 2008). The precise role of adult neurogenesis in the neurobiology of depression, therefore, remains uncertain.

As mentioned above, the glutamate/NO/cGMP/PK-G pathway has also been implicated to play a major role in neuroplasticity (Zarate *et al.*, 2003; Calabrese *et al.*, 2007; Kleppisch & Feil, 2009), as well as in depression- (Zarate *et al.*, 2002b; Zarate *et al.*, 2003; Sanacora *et al.*, 2008)

and anxiety-like (Eroglu & Caglayan, 1997; Volke *et al.*, 1997; Volke *et al.*, 2003a; Volke *et al.*, 2003b) behaviour. As this pathway forms the primary focus of the current study, it will be described in more detail in a separate section.

2.2 Role of the Glu/NO/cGMP/PK-G pathway in depression

Glutamate transmission is strongly implicated in depression (Zarate *et al.*, 2002b; Zarate *et al.*, 2003; Sanacora *et al.*, 2008), and the modulation of the Glu/NO/cGMP/PK-G pathway is being investigated as a mood regulating strategy in a number of ongoing preclinical and clinical studies. Although the majority of work has focussed on the role of glutamate and its receptors, more recent studies have also investigated the more downstream mechanisms of glutamatergic transmission that appear to play a role in mood-regulation.

2.2.1 The role of glutamate in depression

Following release in the synaptic cleft, glutamate may stimulate several postsynaptic receptors. These include N-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid (AMPA) receptors, kainic acid receptors and various classes of metabotropic receptors. An illustration of glutamatergic neurotransmission is provided in Figure 2.2.

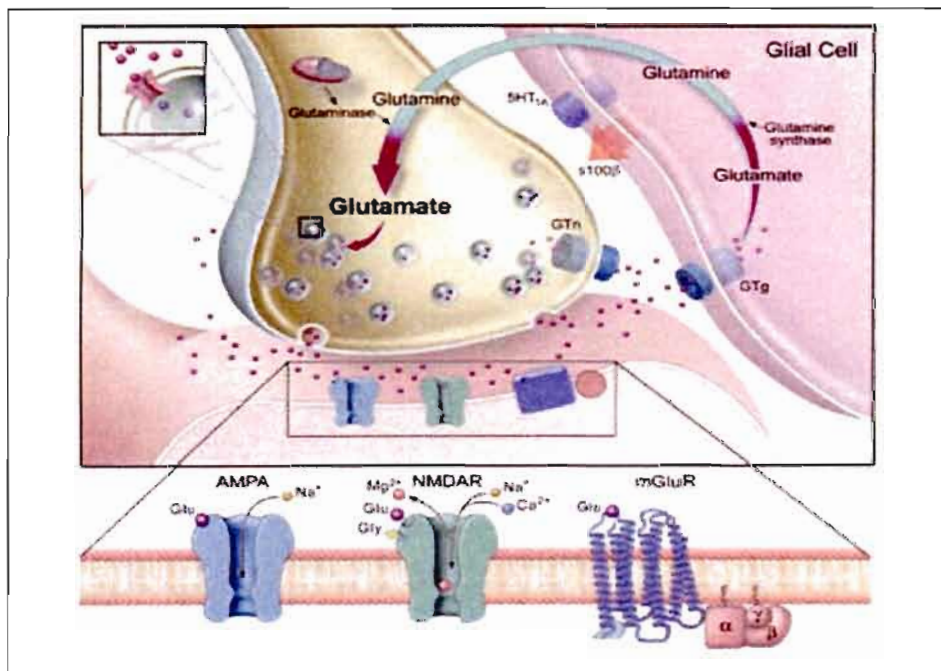


Figure 2.2 Schematic representation of glutamatergic neurotransmission. Following release from the presynaptic terminal, glutamate may bind to various receptors, including ionotropic (NMDA and AMPA) and metabotropic subtypes of glutamate receptors. The actions of glutamate in the synapse are terminated mainly via reuptake mechanisms mediated by glutamate transporters located on presynaptic nerve terminals, as well as on astrocytes (Carlson *et al.*, 2006).

The first substantial evidence for the involvement of glutamate in mood-regulation were from studies that demonstrated that the NMDA receptor antagonist, MK-801, produces antidepressant-like effects in animals (Trullas & Skolnick, 1990), and that tricyclic antidepressants alter the binding properties of NMDA receptors (Reynolds & Miller, 1988). Ever since, an increasing number of preclinical and clinical research reports have suggested that glutamate may be involved in the pathophysiology of mood disorders and antidepressants action (Manji *et al.*, 2003). Clinical studies have shown that drugs that inhibit the release of glutamate are effective in alleviating the symptoms of major depression and/or bipolar disorder, including the anticonvulsant, lamotrigine (Calabrese *et al.*, 1999), and riluzole, a neuroprotective agent with anticonvulsant properties (Zarate *et al.*, 2004). There have also been other studies that have shown antidepressant-like effects for NMDA antagonists, such as MK-801 and AP-7, in various animal models of depression (Zarate *et al.*, 2002a). In fact, it is suggested that the mechanism of action of most antidepressants involve adaptations of NMDA receptor complex functioning (Paul *et al.*, 1994; Skolnick *et al.*, 1996). Interestingly, recent clinical trials have demonstrated that a single dose of the NMDA-receptor antagonist, ketamine, produced a significant improvement in depressive symptoms within a short period of time (72 hours), with the mood elevating effect persisting for 1-2 weeks after the infusion (Zarate *et al.*, 2006).

Another target for glutamate, namely AMPA receptors, are ionotropic receptors that have been implicated in learning and memory processes (Sanderson *et al.*, 2008), whereas drugs that potentiate AMPA receptor function (also referred to as ampakines) have demonstrated antidepressant-like properties in several preclinical models of depression (Li *et al.*, 2001; Black, 2005). These drugs have also been associated with enhanced neurogenesis (Bai *et al.*, 2003) and an increased expression of neurotrophic factors (Lauterborn *et al.*, 2000; Lauterborn *et al.*, 2003). An additional class of glutamate receptors that is currently being investigated for a possible role in mood regulation is the G protein-coupled metabotropic type of glutamate receptors (mGlu receptors) that mediate the slower modulatory actions of glutamate (Zarate *et al.*, 2002a). Preclinical studies suggest that agonists at specific subtypes of mGlu receptor produce antidepressant, anxiolytic and neuroprotective effects (Maiese *et al.*, 2000; Palucha *et al.*, 2004), and that selective agonism/antagonism of various mGlu receptors can induce anxiolytic- and/or antidepressant-like effects (Chojnacka-Wojcik *et al.*, 2001; Tatarczynska *et al.*, 2001).

Taken together, there is convincing preclinical and clinical evidence for the role of glutamate and its receptors in the neurobiology of depression. There are, however, a number of problems in realising glutamatergic drugs as clinically useful antidepressants. It has long been known that the enhancement of glutamatergic transmission leads to excitotoxicity and neuron death (Peterson *et al.*, 1989; Frandsen *et al.*, 1989), whereas neuropsychiatric side-effects are a

problematic side-effect of antagonists at glutamate receptors (Riederer *et al.*, 1991). In addition, the glutamatergic signalling pathway also has important cardiovascular regulatory functions that further complicate the targeting of this system as possible antidepressant target. Therefore, the notion of inducing mood-regulatory actions by the modulation of distal (downstream) targets of the glutamate signal transduction pathway is becoming more attractive, and of particular relevance to the current study is the NO/cGMP/PK-G pathway, a sub-cellular signalling system that is activated upon NMDA receptor stimulation. While this approach is a promising field of ongoing research, preclinical results that have described the role of the NO/cGMP/PK-G pathway in regulating depression- and anxiety-like behaviour are contradictory. The involvement of the NO/cGMP/PK-G pathway in regulating mood will be discussed in § 2.2.3.2.

2.2.2 NO/cGMP/PK-G signalling in the central nervous system

The discovery of NO in the central nervous system as a substance that mediates an increase in the concentration of cGMP following stimulation of NMDA receptors (Garthwaite *et al.*, 1988), has revolutionised our understanding of synaptic transmission and neuronal communication. Since NO is a gas, it readily diffuses from post- to presynaptic terminals, and thereby acts as a retrograde neurotransmitter to regulate presynaptic neuronal function (see § 2.2.2.2). In addition to effects on neuronal transmission and synaptic plasticity (Feil & Kleppisch, 2008; Kleppisch & Feil, 2009), NO signalling has been implicated in the regulation of various cognitive and emotional behaviours in animals (Nelson *et al.*, 1995; Wiley *et al.*, 1995; Dzoljic *et al.*, 1997; Harkin *et al.*, 1999; Heiberg *et al.*, 2002) (see § 2.2.3 below).

2.2.2.1 The physiology of NO/cGMP/PK-G signalling

2.2.2.1.1 Synthesis of NO

Nitric oxide is produced in a reaction in which *l*-arginine is converted to *l*-citrulline and NO via the action of a family of enzymes known as nitric oxide synthases (NOS). Three NOS isozymes have been identified that catalyse this reaction, namely neuronal (nNOS), endothelial (eNOS) or inducible (iNOS) NO synthase (see Figure 2.3). Of these isozymes, nNOS is the most abundantly expressed in the brain and is found in a variety of different neurons (Bredt & Snyder, 1990; Prast & Philippu, 2001). Furthermore, nNOS is calmodulin-dependent and activated via Ca^{2+} influx following the opening of NMDA receptor complex channels (Garthwaite *et al.*, 1988). Another Ca^{2+} /calmodulin-dependent NOS, namely eNOS, is suggested to be confined to endothelial cells, although the synthesised NO is capable of diffusing and signalling to nearby neurons (Garthwaite *et al.*, 2006). On the other hand, iNOS is not detectable in the brain under normal circumstances, and is only expressed following inflammatory stimuli (MacNaul & Hutchinson, 1993).

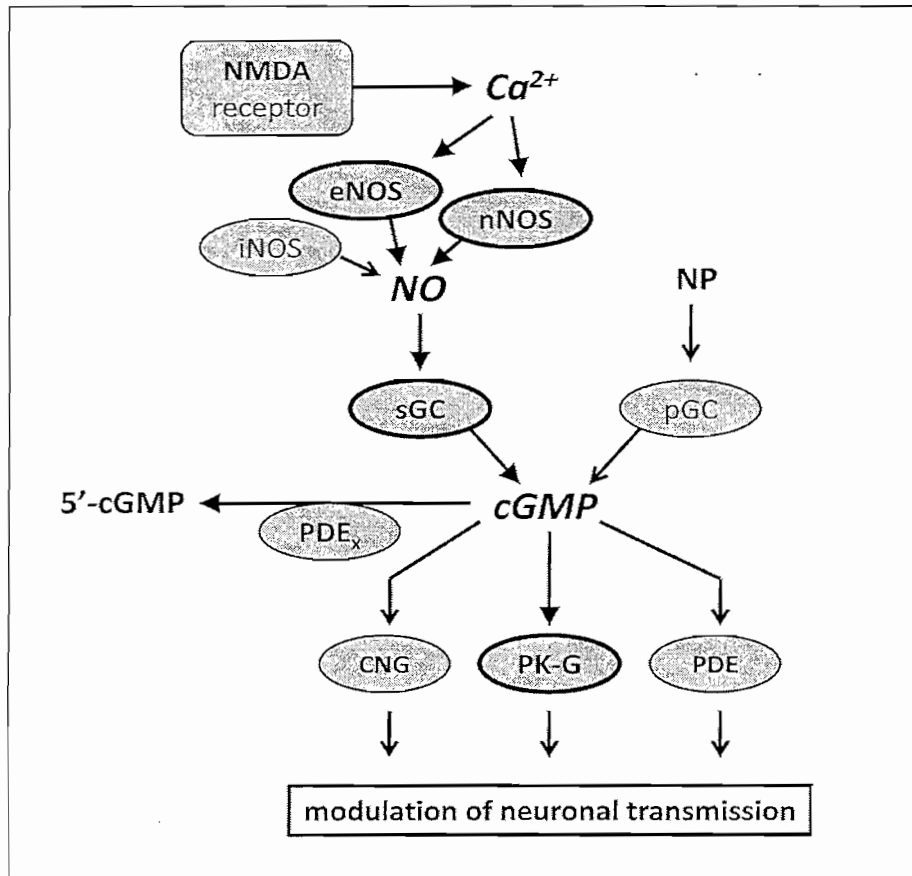


Figure 2.3 The NO/cGMP/PK-G signalling pathway. NO is generated by endothelial (eNOS), neuronal (nNOS) or inducible (iNOS) NO synthase. Activation of nNOS and eNOS is dependent on Ca^{2+} influx following NMDA receptor stimulation by glutamate. A major target for NO is cytosolic soluble guanylyl cyclase (sGC), leading to increased cGMP production and activation of cGMP-dependent protein kinase (PK-G). An increase in cGMP can also be induced by natriuretic peptides via activation of membrane-bound particulate guanylyl cyclases (pGC), whereas cGMP can also signal independently from PK-G by activating cyclic nucleotide-gated ion channels or by modulating the activity of various phosphodiesterases (PDEs). The effects of cGMP are terminated by several selective and non-selective PDEs. Reproduced from Feil and Kleppisch (2008).

2.2.2.1.2 cGMP production in the brain

The principal signalling mechanism of NO appears to be the activation of soluble guanylyl cyclase (sGC) that leads to an increase in the intracellular concentration of cGMP (Figure 2.3) (Friebe & Koesling, 2003). As mentioned above, the majority of neurons in the central nervous system have been shown to be capable of producing NO and/or cGMP (Bredt & Snyder, 1990; Prast & Philippu, 2001), and is in line with the putative role of NO as a universal neurotransmitter in the brain. In addition to the NO-dependent production of cGMP, the natriuretic peptide-stimulated activation of membrane bound particulate guanylyl cyclase (pGC)

is another source of cGMP (Kuhn, 2004), although function of this mechanism in the brain remains largely unknown.

2.2.2.1.3 Targets for cGMP

Known signalling mechanisms for cGMP include the activation of cyclic nucleotide-gated (CNG) ion channels, hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels and several nucleotide hydrolysing phosphodiesterases (PDEs) (see Figure 2.3). However, the major signal transduction pathway of NO and cGMP is believed to involve the activation of cGMP-dependent protein kinase (PK-G) (Hofmann *et al.*, 2009). Two subtypes for PK-G are found in the brain, namely PK-G(I) and PK-G(II) (Feil *et al.*, 2005a), of which PK-G(I) appears to be the most abundant form and expressed in the hippocampus, cerebral cortex, amygdala and various hypothalamic regions to name but a few (Feil *et al.*, 2005b). Several target proteins for PK-G that play a role in synaptic plasticity are summarised in Table 2.2.

In addition to activating PK-G, the activities of several PDEs can also be regulated by cGMP. Together with nucleotide synthesis, this group of enzymes play an integral role in regulating cAMP and cGMP signalling (Sonnenburg & Beavo, 1994). Cyclic GMP is degraded by several cGMP-specific PDEs as well as by non-selective PDEs that hydrolyse both cAMP and cGMP. Importantly, cGMP can inhibit or activate several PDE subtypes and hereby modulate its own concentration and/or that of cAMP. For example, cGMP can increase cAMP signalling by binding to the cGMP-inhibited cAMP-selective PDE3. Cyclic GMP can also lower the levels of both nucleotides by binding to the cGMP-stimulated non-selective PDE2, or decrease its own concentration by binding to the cGMP-selective PDE5 (Feil & Kleppisch, 2008). Therefore, cGMP-regulated PDEs are important cGMP receptors that play a key role in regulating cyclic nucleotide signalling. Particularly, these enzymes are capable of transforming cGMP signals into cAMP signals leading to nucleotide cross-talk in the brain, something that has been termed a Yin-Yang relationship (Harvey *et al.*, 1990a).

Lastly, cGMP can also activate channels. These channels are known to play an important role in the signal transduction pathway of the visual and olfactory systems (Menini, 1995; Reisert & Bradley, 2005) but are also expressed in other neurons where these channels may be involved in the mediation of various functions of NO (Biel *et al.*, 1999; Flynn *et al.*, 2001; Wong & Yao, 2008). However, the specific role(s) of CNG-gated ion channels in the brain are currently poorly understood.

Table 2.2 Substrates for PK-G (reproduced from Feil and Kleppisch (2008))

Substrate	Possible function	Brain region	References
G-substrate	Initiation of cerebellar LTD	Cerebellum	Hall <i>et al.</i> , 1999 Endo <i>et al.</i> , 2003
DARRP-32	Modulation of signalling pathways	Substantia nigra	Tsou <i>et al.</i> , 1993 Nishi <i>et al.</i> , 2005
IP ₃ receptor type I	Stimulation of Ca ²⁺ release from IP ₃ sensitive stores	Cerebellum	Haug <i>et al.</i> , 1999 Wagner <i>et al.</i> , 2003
VASP	Regulation of actin dynamics Vesicle trafficking Aggregation of vesicle proteins	Cerebellum Hippocampus	Hauser <i>et al.</i> , 1999 Arancio <i>et al.</i> , 2001 Wang <i>et al.</i> , 2005
RhoA	Regulation of actin dynamics Vesicle trafficking Aggregation of vesicle proteins	Hippocampus C6 glioma cells Vascular smooth muscle	Wang <i>et al.</i> , 2005 Zhuang <i>et al.</i> , 2004 Sauzeau <i>et al.</i> , 2000
Septin-3	Vesicle trafficking	Hippocampus	Xue <i>et al.</i> , 2000 Xue <i>et al.</i> , 2004
PDE5	Enhanced cGMP degradation	Cerebellum	Shimizu-Albergine <i>et al.</i> , 2003
RGS3 RGS4	Termination of GPCR activation	Astrocytes	Pedram <i>et al.</i> , 2000
RGS2	Termination of GPCR activation Increase transmitter release	Vascular smooth muscle	Tang <i>et al.</i> , 2003
ADP ribosyl cyclase	Decreased transmitter release Initiation of hippocampal LTD	Hippocampus	Fossier <i>et al.</i> , 1999 Reyes-Harde <i>et al.</i> , 1999a Reyes-Harde <i>et al.</i> , 1999b

2.2.2.2 Effects of the NO/cGMP/PK-G pathway on neurotransmitter release

An important discovery regarding the role of NO in the brain was the observation that NO donors influence the release of several neurotransmitters, including acetylcholine, catecholamines and excitatory and inhibitory amino acids (Pogun *et al.*, 1994). Other studies have also demonstrated that the release of acetylcholine, catecholamines, and excitatory and inhibitory amino acids are modulated by endogenous and externally administered NO (Prast & Philippu, 1992; Wegener *et al.*, 2000; Trabace & Kendrick, 2000). It is believed that the release of primarily glutamate is enhanced via a cGMP-dependent mechanism, which then modulates the release of other neurotransmitters in several brain regions, including the hippocampus, hypothalamus, striatum and locus coeruleus (Prast & Philippu, 2001). Importantly, NO can both stimulate (Sporns & Jenkinson, 1997; Stanton *et al.*, 2005) and inhibit (Stanton *et al.*, 2001; Stanton *et al.*, 2003) the release of a particular neurotransmitter, depending on the brain region concerned and NO concentration.

A model of retrograde signalling for NO in a glutamatergic synapse (Feil & Kleppisch, 2008) is illustrated in Figure 2.4. This model proposes that an action potential-induced release of glutamate from presynaptic terminals activates postsynaptic NMDA receptors, leading to the synthesis of NO. The released NO may then diffuse back to the presynaptic terminal, where it stimulates cGMP production and the activation of PK-G, which through the phosphorylation of various target proteins (Table 2.2), can then either increase (as illustrated in Figure 2.4) or decrease neurotransmitter release.

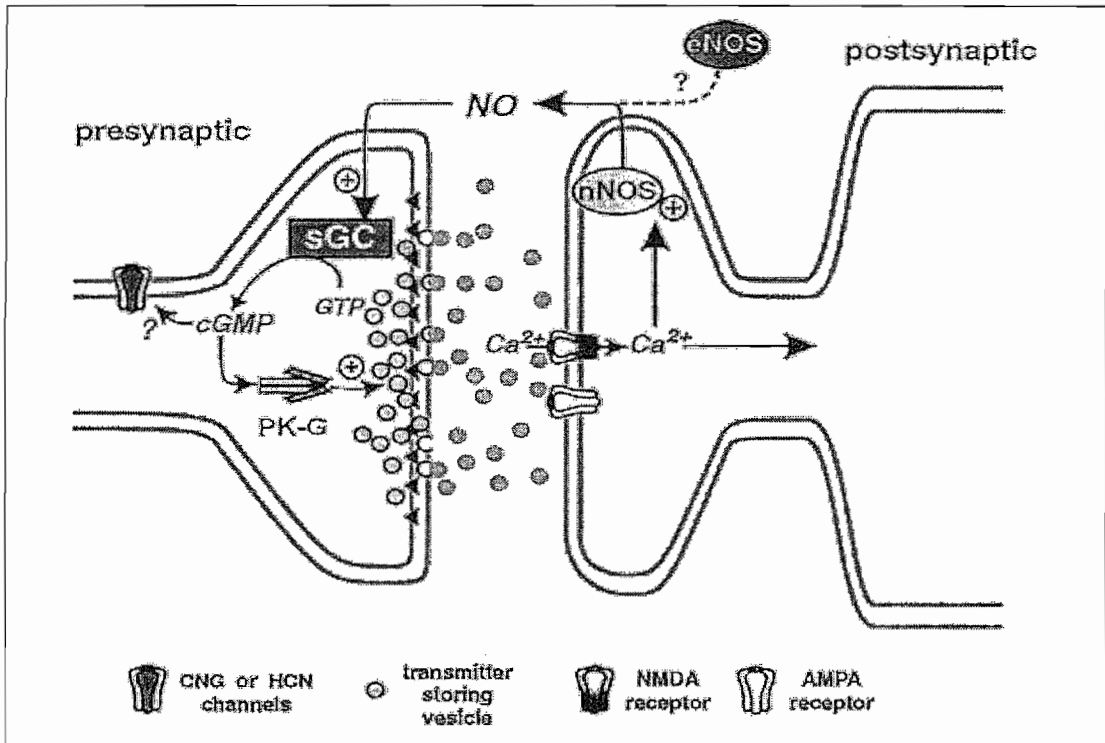


Figure 2.4 Retrograde NO signalling in a glutamatergic synapse. NO is synthesised in the postsynaptic terminal by Ca²⁺/calmodulin-activated nNOS or derived from eNOS in nearby vessels, whereafter it diffuses to the presynaptic terminal and activates sGC. The subsequent increase in intracellular cGMP concentration activates several targets, including PK-G. Through phosphorylation of various target proteins, PK-G ultimately increases presynaptic neurotransmitter release. This is suggested to involve vesicular proteins and other proteins involved in the docking/fusion of vesicles at the release sites (black triangles). Lastly, cGMP may also modulate neurotransmitter release by activating presynaptic nucleotide-regulated ion channels, such as CNG and HCN channels (Feil & Kleppisch, 2008).

Although many studies have now confirmed beyond doubt that NO/cGMP can modulate transmitter release (Prast & Philippu, 2001), the molecular mechanisms and functional relevance of presynaptic NO/cGMP signalling are not well understood. In addition to the targets noted in Table 2.2, other mechanisms are thought to involve PK-G-mediated phosphorylation of vesicle docking proteins, various types of ion channels, and effects on synaptic vesicle recycling (Meffert *et al.*, 1996; Micheva *et al.*, 2003; Schlossmann & Desch, 2009). Nonetheless, the

regulatory action of the NO/cGMP/PK-G pathway on neurotransmitter release may well play a key role in the putative mood-regulating action of this system.

2.2.3 Effects of the NO/cGMP/PK-G pathway on behaviour

2.2.3.1 Learning and memory

The NO/cGMP/PK-G system plays a well-documented role in the induction of long-term potentiation (LTP) and long-term depression (LTD) (Mize *et al.*, 1998), mechanisms that were originally suggested to be an integral component of memory formation by Hebb in 1949. These forms of synaptic plasticity can be described as long-lasting, activity-dependent changes of synaptic transmission that occur in neuronal circuits, and are still believed to function as cellular mechanisms underlying learning and memory (Ito, 2001; Whitlock *et al.*, 2006). Indeed, it has been shown that NO modulates behaviour related to learning and memory processes in animals. For example, inhibition of endogenous NO impairs spatial learning of rats in a radial arm maze (Bohme *et al.*, 1993; Zou *et al.*, 1998), whereas nNOS knockout mice, or rats treated with NOS inhibitors, display impaired performance in the morris water maze and object recognition memory tests (Chapman *et al.*, 1992; Prickaerts *et al.*, 1997; Kirchner *et al.*, 2004; Koylu *et al.*, 2005). Moreover, it appears that the effect of NO on learning and memory is cGMP-dependent, given that several of these responses have been replicated either by administration of a cGMP analogue (Bernabeu *et al.*, 1996) or by an inhibitor of cGMP-selective PDEs (Prickaerts *et al.*, 1997).

2.2.3.2 Affective behaviour

As described in § 2.2.1, there is convincing preclinical and clinical evidence for the involvement of glutamate and its receptors in the neurobiology of depression, and the specific effects of activation or inhibition of glutamate transmission on mood are relatively well described. On the other hand, our understanding of the involvement of the more downstream activation of the NO/cGMP/PK-G pathway is more limited. Preclinical studies have shown that inhibition of this pathway by sGC inhibitors (Eroglu & Caglayan, 1997; Heiberg *et al.*, 2002; Dhir & Kulkarni, 2007; Ghasemi *et al.*, 2008) and NOS inhibitors (Harkin *et al.*, 1999; Heiberg *et al.*, 2002; Harkin *et al.*, 2004; Dhir & Kulkarni, 2007) produces antidepressant-like behavioural responses in rodent models of depression, whereas selective PDE5 inhibitors (thereby increasing cGMP signalling) have been shown to attenuate the antidepressant-like activity of other drugs (Kulkarni & Dhir, 2007; Dhir & Kulkarni, 2007; Jesse *et al.*, 2008; Ghasemi *et al.*, 2008) (a more detailed description of the effects of PDE5 inhibitors on affective behaviour is given in § 2.3.1.2.2 below). In addition, serotonergic antidepressants inhibit NOS activity (Wegener *et al.*, 2003), and a clinical study has reported that depressed patients present with increased plasma nitrite levels,

an end-product of NO metabolism (Suzuki *et al.*, 2001)). Altogether, these studies indicate that the inhibition of the NO/cGMP/PK-G pathway may have an antidepressant action, and that activation of this system may be depressogenic. However, a recent preclinical study from our laboratory suggests that the enhancement of cGMP signalling by the inhibition of PDE5 with sildenafil may have antidepressant properties when combined with a mACh receptor antagonist (Brink *et al.*, 2008) (see § 2.3.1.2.2 below). In addition, studies in humans have also shown that depressed patients present with a reduced number of NOS immunoreactive neurons (Bernstein *et al.*, 2002), as well as reduced NOS activity (Chrapko *et al.*, 2004; Chrapko *et al.*, 2006), supportive of the idea that bolstering the NO/cGMP/PK-G pathway may have beneficial antidepressant actions. There have also been several reports that demonstrated that NO donors and NOS inhibitors display dual effects in rodent models of depression (da Silva *et al.*, 2000; Inan *et al.*, 2004), where the ultimate behavioural responses in these tests were dependent on the doses for the drugs used. Interestingly, this is not different from the dual role that has been described for NO with regard to modulation of neurotransmitter release, since NO can both stimulate and inhibit neurotransmission, depending on the brain region and the concentration in which it is administered (Feil & Kleppisch, 2008).

Taken together, these results form convincing evidence for the involvement of the NO/cGMP/PK-G pathway in the neurobiology of mood-regulation. However, previous clinical and preclinical studies that have investigated the role of the NO/cGMP/PK-G pathway in regulating affective behaviour do not concur on the specific role(s) that this system plays in this regard. This may be reflective of our limited understanding of the mood-regulatory involvement of the NO/cGMP/PK-G pathway in the brain, and prompts further investigation to deepen our understanding on this topic.

2.2.3.3 Anxiety

Several reports have also implicated the NO/cGMP/PK-G system in the regulation of anxiety-like behaviour in animals. Acute administration of NOS inhibitors produces anxiolytic-like effects in the elevated plus maze (EPM) (Volke *et al.*, 1997; Yildiz *et al.*, 2000), the light-dark compartment test (Volke *et al.*, 2003a) and decreases ultrasonic vocalization of rat pups (Campbell *et al.*, 1999). In addition, it has been reported that the augmentation of the NO/cGMP/PK-G pathway via acute inhibition of cGMP-selective PDE5 increases anxiety-like behaviour in mice subjected to an elevated plus maze (Volke *et al.*, 2003b; Kurt *et al.*, 2004) (a more detailed description of the effects of PDE5 inhibitors on anxiety is given in § 2.3.1.2.3 below). However, another study has reported that direct infusion of a NOS inhibitor into the hippocampus or amygdala of rats produced an anxiogenic effect in the EPM (Monzon *et al.*, 2001), whereas an anxiolytic response for an NO donor was observed in mice subjected to the light-dark compartment test (Li & Quock, 2002). Lastly, dual anxiogenic and anxiolytic

responses following the administration of drugs that modulate NO-cGMP activity have been described in rodent models of anxiety (Eroglu & Caglayan, 1997; Spiacci, Jr. *et al.*, 2008).

Therefore, as in the case for the involvement of the NO/cGMP/PK-G system in the regulation of affective behaviour, conflicting evidences for the effects of modulators of this system on anxiety have been reported. The data suggest that the NO/cGMP/PK-G pathway may be involved in both increasing and reducing anxiety, although this may have a dependence on the brain region being studied, and also the dose and duration of treatment of the NO modulating agent being administered (see § 2.3.1.2.3 below).

2.2.4 The involvement of the NO/cGMP/PK-G pathway in the stress response

The NO/cGMP/PK-G pathway has also been suggested to be involved in the response of the brain to psychological stress. Preclinical studies have demonstrated that repeated exposure to inescapable stressors increases hippocampal NOS activity (Harvey *et al.*, 2004) and NO levels (Harvey *et al.*, 2005) in rodents. In addition, a recent study has demonstrated that rats with a genetic predisposition to depression present with increased stress-evoked signalling of the NO/cGMP/PK-G system (Wegener *et al.*, 2009). Since stress is believed to play major role in the aetiology of depression (as discussed in § 2.1.3), this suggests that the NO/cGMP/PK-G pathway may be a genetic factor in determining an increased vulnerability to developing depression.

2.3 The role of phosphodiesterase type 5 in the brain

PDE5 is an enzyme that selectively hydrolyses cGMP, and together with the state of activation of sGC, is an integral regulator of cGMP signalling. The distribution of PDE5 generally coincides with that of PK-G (Puzzo *et al.*, 2008), most likely since these enzymes are both targets for cGMP. In the brain, PDE5 is widely (albeit not uniformly) expressed and includes areas of the cerebellum, caudate nucleus, hippocampus, substantia nigra as well as the subthalamic nucleus (Loughney *et al.*, 1998; van Staveren *et al.*, 2004; Menniti *et al.*, 2006). Of note is that PDE5 is also a substrate for phosphorylation by PK-G, leading to increased cGMP degradation (Thomas *et al.*, 1990), and thereby creating an efficient control mechanism for regulating intracellular cGMP levels.

In the periphery, the principal function of PDE5 is to regulate the state of contraction of blood vessels by modulating cGMP levels in smooth muscle cells (Jackson *et al.*, 1999; Cawley *et al.*, 2007). However, given that PDE5 is also expressed in the brain (described above), and that several preclinical studies have reported central effects for drugs that selectively inhibit PDE5 (see § 2.3.1 below), this enzyme appears to be a promising target for modulating NO/cGMP/PK-

G signalling the brain. Given the abovementioned involvement of the NO/cGMP/PK-G pathway in the regulation of neurotransmitter release and depression- and anxiety-like behaviour (§ 2.2.2), an effective antidepressant strategy may involve the modulation of PDE5 function. Indeed, drugs that selectively inhibit PDE5 have been shown to modify depression-like behaviour in rodents (see § 2.3.1.2.2 below). However, this suggestion has not to date been explored to any great extent.

2.3.1 Selective PDE5 inhibitors

Sildenafil, or more commonly known by its commercial name, Viagra[®], is currently the most widely prescribed treatment for male erectile dysfunction (ED) (Puzzo *et al.*, 2008), and was initially discovered in a search for a novel treatment for arterial hypertension and angina pectoris. In fact, sildenafil is currently also registered for the treatment of pulmonary hypertension (Revatio[®]). In addition to its well-exploited peripheral effects, various studies indicate that sildenafil induces several neurological and behavioural effects (Uthayathas *et al.*, 2007), and accordingly may offer new approaches in the treatment of several neurological and mental disorders.

A number of distinct chemical classes of drugs that selectively inhibit PDE5 have been synthesised. These include cGMP-based drugs such as sildenafil and vardenafil (Levitra[®]), and the β -carboline-derived drug, tadalafil (Cialis[®]). Of these, vardenafil is considered to be the most potent inhibitor, with an IC₅₀ value of approximately 5 times lower for PDE5 compared to sildenafil or tadalafil (Kim, 2003). Importantly, the capability of these drugs to induce central effects following systemic administration depends on their ability to cross the blood-brain barrier. There is clear evidence that sildenafil crosses this barrier (Puzzo *et al.*, 2008). Evidence is still lacking for tadalafil and vardenafil, although several studies have reported central effects for these drugs in animals following systemic administration (Prickaerts *et al.*, 2002; Zhang *et al.*, 2006a; Ko *et al.*, 2009), suggesting that these drugs do enter the brain.

2.3.1.1 Neurological effects of PDE5 inhibitors

Interestingly, several studies have demonstrated that sildenafil (Zhang *et al.*, 2002; Zhang *et al.*, 2006b) and tadalafil (Zhang *et al.*, 2006a) induce an increase in neurogenesis in rats following an embolic model of stroke. In addition, it has also been reported that sildenafil has antinociceptive effects, and therefore may hold promise as a novel treatment strategy for neuropathic pain (Jain *et al.*, 2001; Patil *et al.*, 2004a; Patil *et al.*, 2004b; Ambriz-Tututi *et al.*, 2005; Yoon *et al.*, 2009). It is also believed that PDE5 inhibitors may influence mechanisms of synaptic plasticity such as LTD and LTP (Puzzo *et al.*, 2008), which, as mentioned before, are believed to play a key role in learning and memory processes (Ito, 2001; Whitlock *et al.*, 2006).

The potential for PDE5 inhibitors as memory enhancing drugs is therefore an interesting proposition (see § 2.3.1.2.1 below).

2.3.1.2 Behavioural and cognitive effects of PDE5 inhibitors

2.3.1.2.1 Learning and memory

The majority of preclinical studies that have investigated the behavioural effects of PDE5 inhibitors have explored effects of these drugs on tests of learning and memory. These include preclinical studies that have demonstrated that sildenafil influences long-term memory retention in mice (Baratti & Boccia, 1999). In addition it has also been shown that PDE5 inhibition improves object recognition memory (Prickaerts *et al.*, 2002; Prickaerts *et al.*, 2004; Rutten *et al.*, 2007) and attenuates spatial learning impairments induced by NOS inhibitors (Devan *et al.*, 2006) and antimuscarinic agents in rats (Devan *et al.*, 2004). Another study has shown that sildenafil produces enhancement of memory acquisition and retention in mice tested in the EPM (Singh & Parle, 2003). In humans, one study has shown that sildenafil enhanced the ability to focus attention on streams of auditory stimuli (Schultheiss *et al.*, 2001).

2.3.1.2.2 Affective behaviour

As noted in § 2.2.3.2, preclinical studies that have investigated the involvement of the NO/cGMP/PK-G pathway in depression have reported that sildenafil attenuates the antidepressant-like activity of other drugs in rodent models used for the screening of antidepressant activity (Kulkarni & Dhir, 2007; Dhir & Kulkarni, 2007; Jesse *et al.*, 2008; Ghasemi *et al.*, 2008), which is accord with the well-described antidepressant actions of drugs that inhibit NO-cGMP signalling (Eroglu & Caglayan, 1997; Harkin *et al.*, 1999; Heiberg *et al.*, 2002; Harkin *et al.*, 2004; Dhir & Kulkarni, 2007; Ghasemi *et al.*, 2008). In line with such a depressogenic action, sildenafil has been shown to have a pro-cholinergic effects (Devan *et al.*, 2004; Patil *et al.*, 2004a; Brink *et al.*, 2008), an action that have historically been suggested to cause a worsening of depressive symptoms (Janowsky *et al.*, 1972; Janowsky *et al.*, 1994). However, a worsening of depressive symptoms has not been observed clinically (Tignol *et al.*, 2004). Instead, one study has reported that sildenafil reduces self-reported depressive symptoms in patients treated for erectile dysfunction (Muller & Benkert, 2001).

When taking these points into consideration, it is interesting that recent data from our laboratory has demonstrated that subacute administration of sildenafil induces an antidepressant-like response in the rat forced swim test (FST), but only in combination with a muscarinic acetylcholine receptor antagonist (Brink *et al.*, 2008). These results correspond with the cholinergic hypothesis of depression (§ 2.1.5.2), and suggest that sildenafil has antidepressant activity but that is attenuated by a simultaneous increase in cholinergic neurotransmission. In fact, this cholinotropic action for sildenafil has been demonstrated in *in vitro* cell culture studies

(Brink *et al.*, 2008), whereas other studies have also reported cholinotropic effects for this drug (Devan *et al.*, 2004; Patil *et al.*, 2004a). Therefore, the antidepressant activity of sildenafil may only emerge following an uncoupling of this counteracting depressogenic mechanism. However, whether the antidepressant-like property of sildenafil is shared by other PDE5 inhibitors is currently not known, and it is uncertain whether the activation of PK-G underlies the antidepressant-like response of this drug. Further investigation is therefore needed to clarify these and other aspects of the putative antidepressant potential of PDE5 inhibitors. This forms the primary objective of the current study.

2.3.1.2.3 Anxiety

As noted in § 2.2.3.3, several studies that have investigated the acute effects of PDE5 inhibitors on anxiety have shown that sildenafil increases anxiety-like behaviour in mice subjected to the EPM (Volke *et al.*, 2003b; Kurt *et al.*, 2004). In contrast, a recent study has demonstrated that chronic treatment with sildenafil reduced anxiety-like behaviour of rats in the open field test (Solis *et al.*, 2008). Interestingly, this is not different from the pattern of response observed for fluoxetine and other SSRIs. Acute administration of these antidepressants is known to be anxiogenic, 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 adaptive changes are thought to involve the down-regulation of serotonergic receptors (Harvey, 1997), and the same principle of adaptive response, as well as monoamine involvement, may be responsible for the anxiolytic effect observed for sildenafil following a chronic treatment regime. However, there have been no other studies that assessed the anxiolytic properties of chronic sildenafil in a different animal model of anxiety, or the effects of other PDE5 inhibitors in this regard. The current study aims to address these shortcomings.

2.4 Animal models of depression and screening tests for antidepressant activity

As mentioned in § 2.1.2, the diagnosis of major depression, as defined in the DSM-IV (American Psychiatric Association, 1994), is based on the presence of a set of heterogeneous psychological and physiological symptoms (Table 2.1). This diverse variety of symptoms makes the development of suitable animal models to mimic depression in the laboratory especially challenging. For example, several symptoms of depression, such as suicidal thoughts or excessive feelings of guilt, are not possible to mimic in animal models. However, other symptoms, such as anhedonia, lack of motivation, cognitive and psychomotor symptoms and changes in appetite/weight, do have parallels in animals and can therefore be evaluated in

animal models. This said, several animal models of depression have indeed been developed that are based on certain aspects of measurable symptom parallels, as well as several screening tests that are routinely used to detect antidepressant activity. Three decades ago, McKinney and Bunney (McKinney & Bunney, 1969) developed a set of criteria for animal models of depression. Accordingly, a valid animal model of depression should have the following characteristics as a minimum requirement:

- It should be reasonably similar to the human condition with respect to symptomatology (face validity);
- There should be a behavioural change that can be measured objectively;
- These behavioural changes should be reversible by the same treatments that are effective in humans (predictive validity); and
- The model should be reproducible between different laboratories.

However, unlike other medical conditions of the CNS in which the pathology is well-described, such as Alzheimer's or Parkinson's disease, the underlying neuropathology of depression remains unclear. Therefore, the development of an animal model of depression that is based on accurate criteria of aetiology or biological construct has been a major challenge. A more recent approach to study depression in animals has been to create models that mimic single characteristics or symptoms of depression, instead of attempting to model the syndrome as whole. In 1995, Geyer and Markou (Geyer & Markou, 1995) proposed that the only criteria necessary for the experimental use of an animal model of depression are:

- The model should be responsive to the same treatments that are effective antidepressants in humans (predictive validity); and
- The measured behavioural response should be reliable and robust, and repeatable between laboratories.

It was suggested by these authors that other criteria, such as construct validity (the degree to which the animal model mimics the underlying neurobiology of the disease) may be valuable, but are not essential for the model to have potential as a useful tool for neurobiological research and novel drug discovery. Several animal models have subsequently been developed based on these simplified criteria, and today, these criteria have been instrumental in detecting potential antidepressant-like activity for a number of novel drugs in preclinical settings. It should be noted, however, that some of these tests are not animal models of depression in the conventional sense of the word, since they are not aimed at mimicking the pathological state of human depression. For instance, the forced swim test (§ 2.4.1) and tail suspension test (§ 2.4.2) are only used as screening tools for antidepressant activity. These tests were not

validated for sufficient construct and face validity to be regarded as animal models of depression as such.

2.4.1 Forced swim test (FST)

The forced swim test (FST) was originally developed by Porsolt and colleagues (Porsolt *et al.*, 1977) to detect antidepressant-like activity in rats, and it has also been validated for mice (Porsolt, 2000). The FST is easy to use, reliable across laboratories, and capable of detecting antidepressant activity over a broad range of antidepressants. These properties make the FST the most widely used screening tool for potential antidepressant drugs today.

The FST is based on the observation that rats, following initial escape-directed movements, develop an immobile posture when exposed in an inescapable cylinder of water. When replaced in the swim cylinders 24 hours later, the rats typically revert to the immobile posture more quickly. The immobility behaviour in this test is thought to underlie a failure in persistence to engage in escape-directed behaviour, also referred to as behavioural despair (Lucki, 1997). If antidepressant drugs are administered between the two swim sessions, the rats will persist in engaging in escape-directed movements for longer periods of time compared to vehicle-treated controls.

A disadvantage of the original (unmodified) FST is that this test is unreliable in detecting the antidepressant-like properties of SSRIs (Lucki, 1997), although these drugs are well-known to be clinically effective antidepressants. A publication by Cryan and colleagues in 2002, described a modified version of the swim test in an effort to render this model more sensitive to the antidepressant activity of SSRIs (Cryan *et al.*, 2002). The procedural modifications included increasing the water depth, as well as using a time sampling technique in which the predominant behaviour during a 5 second interval was scored. This modification enabled researchers to measure three distinct types of behaviour during a single FST session (illustrated in Figure 2.5):

- **Climbing** (or struggling) behaviour is defined as upward-directed movements of the forepaws along the inside of the swim cylinder;
- **Swimming** behaviour is defined as horizontal movements throughout the cylinder that includes crossing into another quadrant; and
- **Immobility**, that is defined, as in the traditional Porsolt test, when no active movements are made, except those that are necessary to keep the rat's head above the water.

A very useful consequence from these modifications is that the modified FST is capable of distinguishing between noradrenergic and serotonergic mechanisms of antidepressant drugs (Cryan *et al.*, 2002). Although antidepressants in general cause a reduction in immobility, drugs

that enhance noradrenergic neurotransmission induce a selective increase in climbing, whereas enhancers of serotonergic neurotransmission selectively increase swimming behaviour (Cryan *et al.*, 2002). However, a major shortcoming of the FST (original or modified) remains that it is responsive to short-term antidepressant treatment, in contrast to the extended (weeks) duration of treatment that is usually necessary for a response in humans.

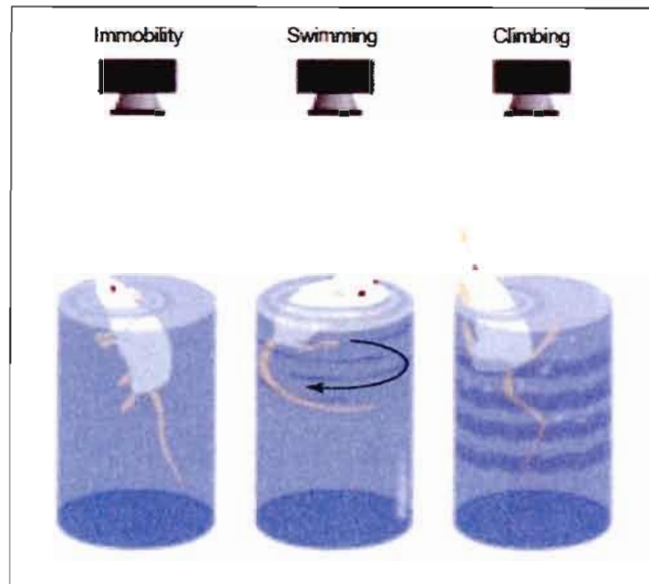


Figure 2.5 Behavioural components in the modified FST.

In this test, effective antidepressant drugs generally reduce immobility, whereas serotonergic mechanisms induce an increase in swimming, and noradrenergic mechanisms increase the time spent engaging in swimming behaviour.

Reproduced from Cryan *et al.* (2002).

2.4.2 Tail suspension test

Like the FST, the tail suspension test is one of the most widely used tests for the detection of antidepressant-like activity, but more specifically in mice (Cryan *et al.*, 2005). In close relation to the FST, this test is based on the observation that mice, when exposed to an inescapable stressor (in this case being suspended by their tail) will eventually revert to an immobile posture. A wide spectrum of acutely administered antidepressants reduces the time spent in this immobile state and promotes escape-directed movements (Cryan *et al.*, 2005). Although the tail suspension test has considerable value in terms of detecting antidepressant-like behaviour, it is suggested that this test is best employed as a component of a battery of several different animal models of depression (Cryan *et al.*, 2005).

2.4.3 Sucrose preference test

This test was first described by Willner *et al.* (1987), where these authors reported that rats displayed a reduced preference for saccharin or sucrose solutions following exposure to several weeks of chronic unpredictable mild stress. Since rats normally exhibit a marked preference for sweet tasting solutions, this response is believed to reflect a decreased interest or pleasure (also referred to as anhedonia) that is often experienced by patients with major depression. Important characteristics of this test are that the depression-like behaviour persists for several weeks following exposure to chronic mild stress, and that antidepressant drugs are able to reverse this deficit only following chronic (2-4 weeks) treatment.

2.4.4 Olfactory bulbectomy

Following a bilateral bulbectomy, rats present with a complex array of behavioural, neurochemical, endocrinological and immunological features that correlate with changes seen in human depression (Kelly *et al.*, 1997). Although the aetiological and construct validity of olfactory bulbectomy as an animal model of depression is clearly questionable, it has been demonstrated that this model has a very accurate capability to detect drugs with known antidepressant activity (strong predictive validity), and is reliable and repeatable between laboratories (Kelly *et al.*, 1997). In addition, this model reveals an antidepressant-like response only following chronic treatment (Kelly *et al.*, 1997), which is a particularly valuable attribute.

2.4.5 Learned helplessness

The learned helplessness paradigm is based on the observation that, following repeated uncontrollable foot shocks, animals display a deficit in making use of an easily accessible escape route (Weiss & Kilts, 1998). This deficit is reversed by a broad spectrum of antidepressant treatments that are acutely administered (Weiss & Kilts, 1998), and by incorporating a form of chronic mild stress into this paradigm, this test has recently been modified to yield a model that is only responsive following chronic treatment (Gambarana *et al.*, 2001), and thus more closely matches the situation in humans.

2.4.6 Flinders Sensitive Line rat: a genetic rat model of depression

The Flinders Sensitive Line (FSL) rat was initially developed in an attempt to create a line of rats that was genetically more resistant to the effects of the cholinesterase inhibitor, diisopropyl fluorophosphate (DFP). Instead, selective inbreeding led to a strain of rat that was genetically more sensitive to the effects of this cholinergic agent (Russell & Overstreet, 1987). This strain was subsequently named the Flinders Sensitive Line (FSL) rat strain, and present with several behavioural, neurochemical, and pharmacological features that have also been reported in depressed individuals (Overstreet *et al.*, 2005b), and has also been used for detecting

antidepressant activity. The behavioural features of these animals that are related to depression include changes in appetite, psychomotor function, sleep patterns and immune function, whereas FSL rats also have pronounced serotonergic and cholinergic abnormalities (Overstreet *et al.*, 2005b), consistent with the suggested involvement of these systems in the neurobiology of depression (see § 2.1.5). Another important validity aspect of this model is that known antidepressants reduce immobility in the FST in these rats following chronic, but not acute treatment (Overstreet, 1993; Overstreet *et al.*, 1995a; Dremencov *et al.*, 2004). In efforts to enhance the aetiological validity of this model, recent studies have superimposed the genetic vulnerability of FSL rats with subacute stress (Wegener *et al.*, 2009) as well as an early-life trauma (El Khoury A. *et al.*, 2006) paradigms. Not only do these stressors induce depression-like behavioural alterations in FSL rats (see below), but neurobiological changes that are associated with depression have also been reported (Petersen *et al.*, 2008; Husum *et al.*, 2008; Wegener *et al.*, 2009; Ryan *et al.*, 2009), thereby providing increased construct validity for this model. Since FSL rats were used to meet some of the objectives in the current study, the key behavioural and neurobiological characteristics of these rats will be described in more detail.

2.4.6.1 Behavioural characteristics

Affective behaviour

A key behavioural characteristic of FSL rats is that they exhibit an inherent increase in immobility in the FST compared to the control line, the Flinders Resistant Line (FRL) rats (Overstreet, 1993). Whereas a 2-day exposure (exposure to a pre-swim session 24 hours prior to the final test session) is employed in the classical FST, a pre-swim session is not required for the behavioural deficit (increased immobility in the FST) to be evident in FSL rats relative to FRL control rats (Overstreet, 1993; Dremencov *et al.*, 2004; Overstreet *et al.*, 2004). Importantly, the increased immobility exhibited by FSL rats is only normalised following chronic (usually 2 weeks) treatment. Other behavioural characteristic of FSL rats that may be representative of human depression includes abnormalities in hedonic responses. Although the preference for a saccharin solution in these rats was found not to differ from FRL controls under baseline conditions, exposure to a chronic mild stress paradigm induced a more pronounced anhedonic response in FSL rats (Pucilowski *et al.*, 1993). As mentioned above, exposure of FSL rats to an early-life maternal separation paradigm has been shown to induce an exaggerated increase in immobility in FSL rats compared to FRL control rats in the FST (El Khoury A. *et al.*, 2006). Similarly, FRL rats subjected to a mild sub-chronic stressor do not show an increase in glutamate-NO signalling in the hippocampus, while a hypersensitive glutamate-NO pathway is evident in FSL rats under the same conditions, suggesting the this pathway may be a genetic factor determining an increased vulnerability to developing depression (Wegener *et al.*, 2009)(Wegener *et al.*, 2009) (also noted in § 2.2.4 above). Since it has been suggested that

human depression may underlie a strong connection between genetic and environmental factors (i.e. stress) (discussed in § 2.1.3), the modelling of this gene X environment interaction shows promise for a model with enhanced aetiological validity.

Anxiety

Initially, it was believed that the FSL rat model was a pure model of depression since these rats did not show increased anxiety-like behaviour relative to FRL rats in the EPM (Overstreet *et al.*, 1995b). However, subsequent studies have now revealed that FSL rats indeed present with increased anxiety-like behaviour in the social interaction test (Overstreet *et al.*, 2004) as well as in the active avoidance task (Overstreet *et al.*, 1990), both of which have been suggested to be indicative of increased anxiety. Therefore, FSL rats may also hold potential as a useful animal model of anxiety.

2.4.6.2 Neurobiological characteristics

Cholinergic abnormalities

As described in § 2.1.5.2, the cholinergic hypothesis of depression was based on the cholinergic-adrenergic imbalance model of depression and mania, as proposed by Janowsky and co-workers (Janowsky *et al.*, 1972). The same group later suggested that depressed individuals exhibit a cholinergic supersensitivity, which could be observed as an exaggerated behavioural or hormonal response to cholinergic agonists (Janowsky *et al.*, 1994). Given that FSL rats are also more sensitive to cholinergic agonists (Overstreet & Russell, 1982; Overstreet, 1993; Overstreet *et al.*, 2005b), the FSL rat model is consistent with the cholinergic model of depression. However, it has been noted more recently that the cholinergic abnormalities of FSL rats may not underlie the key depression-like characteristics of this strain. For instance, anticholinergic drugs do not produce antidepressant-like effects in FSL rats (Overstreet *et al.*, 1995a), and drugs that are known to alter cholinergic neurotransmission (DFP or exposure to lithium) do not alter immobility time in the FST (Overstreet, 1993; Overstreet, 2002). This is in line with the general lack of efficacy of anticholinergic drugs in the treatment of depression.

Serotonergic abnormalities

Several studies have reported serotonergic abnormalities in FSL rats (Wallis *et al.*, 1988; Overstreet *et al.*, 1994; Zangen *et al.*, 1997). For instance, FSL rats are more sensitive to the hypothermic effects of selective 5-HT_{1A} receptor agonists (Wallis *et al.*, 1988; Overstreet *et al.*, 1994), whereas high concentrations of serotonin have been reported in the limbic areas of FSL rats relative to that of FRL controls, and is normalised following chronic antidepressant treatment (Zangen *et al.*, 1997). However, although FSL rats display several serotonergic abnormalities, it is unknown whether these differences reflect the neuropathology of depression

in humans (Overstreet *et al.*, 2005a). It is especially difficult to relate serotonergic alterations in FSL rats to measurements in patients with major depression, since both increases and decreases in serotonergic activity have been reported in clinical studies (Mikuni *et al.*, 1991; Lesch, 1991; Arango *et al.*, 1995). Lastly, an interesting observation regarding the serotonergic abnormalities of FSL rats is that these rats present with a deficiency in the release of dopamine following direct infusion of serotonin into the nucleus accumbens, compared to FRL control rats (Zangen *et al.*, 2001). This abnormal serotonin-induced accumbal dopamine response is normalised by chronic antidepressant treatment, and also more rapidly so by nefazodone, an antidepressant that has been suggested to have a more rapid onset of antidepressant action (Dremencov *et al.*, 2004).

2.5 Synopsis

In summary, depression is a disease that poses serious problems for the population of South Africa, the rest of Africa, as well as the rest of the world. Depression is a complex, multifaceted disease that most likely involves various neuronal circuits and regions of the brain. Although the vast majority of antidepressants in use today remain drugs that target monoaminergic neurotransmission, these approaches have drawbacks with regard to onset of action, treatment resistance and side effects. More recent investigations have revealed that the NO/cGMP/PK-G pathway may hold promise as a target for novel antidepressant drug development. However, considering the widespread distribution of this pathway throughout the body, targeting either NOS or sGC is met with major concerns regarding the risk of cardiovascular and neuropsychiatric toxicity. Nevertheless, selective inhibitors of PDE5 appear to be promising drug candidates for the modulation of the NO/cGMP/PK-G pathway in the treatment of mental disorders. It is our intent to demonstrate that this group of drugs may represent a new approach to treating mood disorders, particularly depression. The specific involvement of NO/cGMP/PK-G signalling and PDE5 inhibition in regulating mood remains somewhat controversial, and this study will also provide new knowledge in this regard. Lastly, animal models of depression are a useful preclinical screening tool for detecting antidepressant activity of novel drugs. The Flinders Sensitive Line (FSL) rat, a genetic rat model of depression, displays improved aetiological validity and is a useful tool for evaluating antidepressant- and anxiolytic-like activity of drugs. This model has not yet been used to assess the antidepressant-like properties of PDE5 inhibitors, which is another important outcome of this study.

Manuscript A

Chapter 3

In this chapter, a manuscript titled

*“Antidepressant-like properties of phosphodiesterase 5 inhibitors
in a genetic rat model of depression”*

is presented. The paper was 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 B). The references for this manuscript are provided at the end of this chapter.

This manuscript has been provisionally accepted for publication on the precondition of some minor changes.

TITLE PAGE**Full title**

Antidepressant-like properties of phosphodiesterase 5 inhibitors in a genetic rat model of depression

Running title

Antidepressant properties of PDE5 inhibitors

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ABSTRACT

We explored the antidepressant-like properties of phosphodiesterase 5 (PDE5) inhibitors in a genetic animal model of depression, namely Flinders Sensitive Line (FSL) rats. We also investigated the dose-dependency of the antidepressant-like action of sildenafil, and its interaction with the cholinergic system and behavioural correlates of monoaminergic neurotransmission, in the forced swim test (FST). Any antidepressant-like properties of tadalafil (a structurally distinct PDE5 inhibitor) was also evaluated. FSL rats were treated for 14 days with vehicle, fluoxetine, atropine or PDE5 inhibitors \pm atropine. Immobility, swimming and climbing behaviours were assessed in the FST. In combination with atropine, both sildenafil (10, 20 mg/kg) and tadalafil (10 mg/kg) decreased immobility while increasing swimming (serotonergic) and climbing (noradrenergic) behaviours. Interestingly, sildenafil (3 mg/kg) decreased immobility while selectively increasing climbing behaviour in the absence of atropine. These results suggest that the antidepressant-like activity of PDE5 inhibitors involve alterations in monoaminergic neurotransmission, as well as dependence on inherent cholinergic tone, so that the final response may be determined by the relative extent of activation of these systems. Furthermore, the antidepressant-like properties of sildenafil are dose-dependent with respect to its interaction with the cholinergic system, as well as in the behavioural profile displayed in the FST.

Keywords: Flinders sensitive line rat, sildenafil, tadalafil, forced swim test, phosphodiesterase 5, cholinergic, Nitric oxide, cGMP.

INTRODUCTION

Whereas the monoaminergic hypothesis of depression and refined versions thereof have dominated our understanding of the neurobiological basis of depression and of antidepressant action, newer hypotheses have explored a role for neuroplasticity in depression (Manji et al., 2003). While the monoaminergic and more recent hypotheses are not mutually exclusive, these latter theories have rather aimed to be more inclusive of diverse observations associated with the neuropathology of depression. These observations include the modulation of pathways associated with neuroplasticity and of anatomical and histological changes observed in the brains of patients after severe long-term depression, or animals after severe stress, most of which may be reversible or preventable by antidepressants (Manji et al., 2003). In this regard the NO-cGMP pathway appears to play a pivotal role (Suzuki et al., 2001; Suzuki et al., 2003; Chrapko et al., 2004; Oliveira et al., 2008), particularly with regard to its neuroregulatory actions on neurotransmitter release (Prast and Philippu 2001; Feil and Kleppisch 2008), as well as being involved in neuroplasticity (Manji et al., 2003; Feil and Kleppisch, 2008; Serulle et al., 2008). Moreover, depression is known to follow chronic stressful environmental conditions (Kendler et al., 2001), whereas stress activates NO synthase (NOS) (Harvey et al., 2005; Joca et al., 2007). Pre-clinical studies have provided evidence for the antidepressant-like properties of guanlyl cyclase - and NOS inhibitors (Heiberg et al., 2002; Harkin et al., 2004) as well as the ability of known antidepressants to inhibit NOS (Wegener et al., 2003), thereby providing a clear rationale for investigating the NO-cGMP pathway as a novel therapeutic target in depression. The majority of these studies report antidepressant-like effects following inhibition of the NO-cGMP pathway; however, there have been several studies suggesting dual effects for NO donors and NOS inhibitors in rodent

models of depression, where the outcome of the response was dependent on the doses used for these drugs (da Silva et al., 2000; Inan et al., 2004).

Sildenafil is a selective inhibitor of phosphodiesterase type 5 (PDE5), the enzyme responsible for cGMP degradation, thereby enhancing cGMP signalling (Puzzo et al., 2008). In addition to its well-exploited relaxing effect on smooth muscle in the clinical treatment of erectile dysfunction and pulmonary hypertension, more recent investigations into the significance of NO-cGMP signalling in the brain and its role in various cognitive and emotional processes, have also revealed central effects for sildenafil, including increased neurogenesis (Zhang et al., 2002; Zhang et al., 2006b), improved object recognition memory (Prickaerts et al., 2002; Prickaerts et al., 2005) and angiogenic effects (Volke et al., 2003; Kurt et al., 2004) in rodents. An enhanced ability to focus attention has also been demonstrated in humans (Schultheiss et al., 2001).

Recently we demonstrated an antidepressant-like effect for sildenafil in Sprague-Dawley rats that is dependent on simultaneous treatment with a muscarinic acetylcholine (mACh) receptor antagonist (atropine) (Brink et al., 2008). Given that sildenafil has inherent cholinotropic actions (Devan et al., 2004; Patil et al., 2004; Brink et al., 2008), and that increased cholinergic tone is associated with depression (Janowsky et al., 1972), we proposed that its antidepressant-like activity may only be revealed following uncoupling of, or attenuating, muscarinic drive. Paradoxically, other studies report an inhibitory action for sildenafil on the antidepressant-like activity of other drugs in animal models of depression (Dhir and Kulkarni 2008; Kulkarni and Dhir 2008). However, these manifestations have not been observed clinically (Tignol et al., 2004). Instead, it has been reported that sildenafil reduces self-reported depressive symptoms in patients treated for erectile dysfunction (Muller and Benkert 2001).

The present study set out to evaluate whether the previously observed antidepressant potential of sildenafil (Brink et al., 2008) can be replicated in a pathological model of depression in which rats are genetically more susceptible to depressive-like behaviour, and also whether this response is common to other PDE5 inhibitors. Flinders Sensitive Line (FSL) rats are innately more immobile in the FST than Flinders Resistant Line (FRL) control rats, and exhibit a decrease in immobility following chronic, but not acute, antidepressant treatment (Overstreet 1993; Overstreet 2002), therefore more closely resembling the clinical scenario in humans. Given previous evidence for dualistic dose-dependent actions by NO donors in the FST (da Silva et al., 2000; Inan et al., 2004), and our hypothesis of multiple activation of pro- and antidepressant mechanisms by sildenafil, we also investigated the dose-dependency of the antidepressant-like properties of sildenafil in the FST. Immobility, swimming and climbing behaviours were assessed, parameters that are indicative of antidepressant-like activity, as well as of the involvement of serotonergic and noradrenergic mechanisms, respectively (Cryan et al., 2002). Lastly, in order to determine whether the antidepressant-like property of sildenafil is a property unique to this drug, or whether it may be related to its PDE5 inhibitory action, shared by other PDE5 inhibitors, we evaluated the possible antidepressant-like properties of tadalafil, a structurally unrelated PDE5 inhibitor \pm atropine in FSL rats in the FST.

MATERIALS AND METHODS

Animals. Male Flinders sensitive line (FSL) rats, and a corresponding negative control line, the Flinders resistant line (FRL) rats, 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 (approval numbers: NWU-00009-07-A9 and NWU-00010-07-A0), 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 administered at doses that were previously used for antidepressant-like or central effects in rats, i.e. fluoxetine (5 mg/kg, a kind gift from Aspen, Port Elizabeth, South Africa) (Overstreet and Griebel 2004), atropine (1 mg/kg, Merck, Darmstadt, Germany) (Brink et al., 2008), sildenafil (1, 3, 10, 20 mg/kg, a kind gift from Pfizer Global Research and Development, Kent, United Kingdom) (Prickaerts et al., 2002; Brink et al., 2008), tadalafil (10 mg/kg, a kind gift from Eli Lilly and Company, Indianapolis, IN, United States of America) (Zhang et al., 2006a). All drugs were dissolved in vehicle containing 5% dimethylsulfoxide (DMSO) in saline, with rats treated chronically with either vehicle (control) or vehicle plus drug(s) via single daily intraperitoneal injections (injection volume = 0.5 ml) for 14 consecutive days. The FRL rat line was used as a behavioural negative control to validate the established model in our laboratory (i.e. to demonstrate lower immobility compared to FSL rats). Consequently, one group of FRL rats ($n = 12$) receiving vehicle for 14 days was included in the study.

Behavioural testing. Approximately 12 hours following the final injection and 1 hour after the start of the dark cycle (19:00), the rats were subjected sequentially to the open

field test (for measurement of locomotor activity) and 2 hours thereafter, to the FST. Observations and analyses of data using rats that were subjected to the FST only, indicated that exposure to the open field test does not affect behaviour in the FST (data not shown). This has also been confirmed previously (Overstreet and Griebel 2004).

The open field test was carried out as described previously (Overstreet and Griebel 2004). Rats were placed into a square test arena (1 m², marked with sixteen 25×25 cm blocks), and their behaviour recorded on video for 5 minutes. The total number of line crossings was scored as a measure of locomotor activity.

The forced swim test was performed as described previously for FSL rats (Overstreet and Griebel 2004; Dremencov et al., 2004). In these studies, a pre-swim session was not used, as would be the case when using rodents without inbred enhanced immobility, such as Sprague-Dawley rats (Cryan et al., 2002). Although some researchers have used a pre-swim session when working with FSL rats (Petersen et al., 2009), this is not common practice. Rats were placed into plexiglass cylindrical tanks (40 cm high and 18 cm in diameter) containing 18 cm of water maintained at 23°C, and behaviour recorded on video under red light illumination for 5 minutes. Rats were scored as immobile when only the necessary movements were made to keep their heads above the water. Swimming was defined as horizontal movements throughout the swim cylinder that includes crossing into another quadrant, and climbing as upward-directed movements of the forepaws along the side of the swim chamber (Cryan et al., 2002).

Statistical analysis. The data for immobility, swimming, climbing and locomotor activity were analysed using one-way analyses of variance (ANOVA) followed by Tukey-Kramer post-hoc analyses (GraphPad Prism® version 5.00, San Diego California, U.S.A.). The data were pooled and analysed in the groupings presented in

the figures, and separate control groups were used for each analysis. Data are expressed as the mean + SEM, and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Fig. 1a illustrates the deficit in escape-directed behaviour observed in FSL rats compared to FRL controls, given the significantly elevated immobility of the FSL strain as measured in the FST following vehicle treatment [$F(2,45) = 27.68, P < 0.0001$], which was normalized (i.e. comparable to FRL rats) following chronic treatment with fluoxetine. Swimming behaviour was significantly lower in vehicle-treated FSL rats compared to FRL controls, whereas treatment of FSL rats with fluoxetine increased swimming to a level that was no longer significantly different from vehicle treated FRL rats [$F(2,45) = 39.18, P < 0.0001$; Fig. 1b]. There were no significant differences in climbing behaviour between vehicle-treated FSL rats, fluoxetine-treated FSL rats or vehicle-treated FRL rats [$F(2,45) = 1.84, P = 0.17$; Fig. 1c].

In Fig. 2a it can be seen that chronic treatment of FSL rats with atropine, sildenafil alone at doses of 1, 10 or 20 mg/kg, or sildenafil at 1 mg/kg in combination with atropine, did not significantly alter immobility times compared to vehicle-treated FSL control rats [$F(9,127) = 8.85, P < 0.0001$]. On the other hand, sildenafil alone at a dose of 3 mg/kg, as well as sildenafil at doses of 3, 10 and 20 mg/kg + atropine significantly decreased immobility compared to vehicle treated FSL control. Swimming behaviour was unaltered by atropine and sildenafil alone, but was significantly increased by sildenafil (10, 20 mg/kg) + atropine compared to vehicle-treated FSL control [$F(9,127) = 5.79, P < 0.0001$; Fig. 2b]. As can be seen in Fig. 2c, sildenafil alone at 3 mg/kg significantly increased climbing [$F(9,127) = 5.07, P < 0.0001$] compared to vehicle-treated FSL control. In combination with atropine, sildenafil increased climbing at all doses except at 1 mg/kg, compared to vehicle-treated FSL rats.

Fig. 3 shows that tadalafil (10 mg/kg/day) alone did not alter immobility, swimming or climbing times compared to vehicle-treated FSL control. However, in combination with

atropine, it evoked a significant decrease in immobility [$F(2,45) = 11.6, P < 0.0001$; Fig. 3a], and increases in swimming [$F(2,45) = 3.89, P = 0.028$; Fig. 3b] and climbing [$F(2,45) = 4.94, P = 0.012$; Fig. 3c] behaviours compared to vehicle-treated FSL rats. Locomotor activity as measured in the open field test was not significantly altered by any of treatments compared to vehicle-treated FSL control [$F(12,112) = 1.07, P = 0.40$; data not shown], and differences in immobility in the FST can therefore not be attributed to treatment-induced alterations in locomotor activity.

DISCUSSION

We have validated the FSL rat model of depression in our laboratory by demonstrating increased immobility in FSL relative to FRL rats in the FST, which is restored to control levels following chronic treatment with fluoxetine, a selective serotonin inhibitor (Fig. 1a). In addition, FSL rats show impaired swimming behaviour compared to FRL control rats (Fig. 1b), suggestive of a serotonergic deficit (Cryan et al., 2002), which coincides with other studies suggesting serotonergic abnormalities in FSL rats (Overstreet et al., 1994; Zangen et al., 1997). In line with its serotonergic mechanism, fluoxetine significantly increases swimming (Fig. 1b), but not climbing behaviour (Fig. 1c) in the FST.

The current study confirms the antidepressant-like properties of sildenafil in combination with a muscarinic acetylcholine (mACh) receptor antagonist (atropine), in a genetic animal model of depression. Interestingly, the dependency of the antidepressant-like effect of sildenafil on concurrent mACh receptor blockade appears to be strongly dose-related, since a lower dose of sildenafil (3 mg/kg) is effective in reducing immobility in the absence of atropine (Fig. 2a), although a dose of 1 mg/kg appears to be insufficient for any response to be observed. Therefore, at 3 mg/kg sildenafil may activate antidepressant-like mechanism(s) in the absence of an attenuating increased cholinergic tone. On the other hand, higher doses of sildenafil require co-administration of atropine for activity in the FST, suggestive of an inhibitory cholinergic mechanism at these doses. Effects on differential neurotransmitter release following PDE5 inhibition may explain some of these dose-dependent observations. Several reports have now confirmed a role for the NO-cGMP pathway in modulating the release of various transmitters in the brain, especially in the hippocampus, a brain region of importance in the neurobiology of depression - see Prast and Philippu (2001) for a review. Specifically, evidence for NO-cGMP-stimulated release of acetylcholine

(Prast and Philippu 1992; Prast et al., 1995) and norepinephrine (Jones et al., 1995; Satoh et al., 1996) have been reported, as well as a biphasic effect on serotonin release (Kaehler et al., 1999). Therefore, the ultimate behavioural outcome of sildenafil treatment may depend on the collective state of neuronal functioning, with antidepressant-like effects only being expressed when mood-elevating mechanism(s) are dominant. This may include states where the choice of dose allows for selective activation of antidepressant mechanism(s), or following selective blockade of depressive (cholinergic) counterparts that will leave the antidepressive systems unattenuated.

As described previously (Cryan et al., 2002), increases in swimming and climbing behaviour in the FST are associated with serotonergic and noradrenergic antidepressant mechanisms, respectively. At higher doses, chronic sildenafil in combination with atropine increases both swimming and climbing behaviour in the FST (Fig. 2). However, a lower dose of sildenafil alone (3 mg/kg) evoked a selective increase in climbing, which may be indicative of a higher affinity of sildenafil for noradrenergic-like mechanisms relative to serotonergic-like and cholinergic targets. Furthermore, recent unpublished data from our laboratory has demonstrated that sub-acute treatment with sildenafil (10 mg/kg) + atropine increases climbing, but not swimming behaviour in the FST. Indeed, sildenafil has been found to have antinociceptive activity (Yoon et al., 2009), an effect it shares with noradrenergic antidepressants such as duloxetine (Iyengar et al., 2004). Together, these results suggest that noradrenergic-associated mechanisms may be the primary site of action for sildenafil at lower doses, whereas serotonergic-like responses are only activated following chronic (2 weeks) treatment and at higher doses (10 and 20 mg/kg), but are subject to cholinergic modulation (Fig. 2b). In agreement with this, another study has demonstrated a biphasic action for NO-donors on the serotonergic system, where high

doses of these drugs were associated with increased serotonin release (Kaehler et al., 1999). It should be noted, however, that mechanisms other than presynaptic modulation of transmitter release may also be involved in the antidepressant-like action of sildenafil. Modulation of postsynaptic neuronal functioning as well as regulatory actions on gene expression have been described following activation of the NO-cGMP system (Kleppisch and Feil 2009).

In the final component of this study, we provide evidence that the antidepressant-like properties of sildenafil may be shared by other PDE5 inhibitors and related to the inhibition of PDE5. In this regard, tadalafil, a selective PDE5 inhibitor that is structurally distinct from sildenafil, evoked a similar response pattern in the FST to sildenafil, including dependency on atropine. Chronic treatment of FSL rats with tadalafil + atropine reduces immobility (Fig. 3a), while increasing swimming (Fig. 3b) and climbing (Fig. 3c) behaviours in the FST. As with sildenafil (10 mg/kg) + atropine, these results suggest that the tadalafil + atropine combination may exert an anti-depressant-like effect via the enhancement of both serotonergic and adrenergic mechanisms. It has to be noted, however, that the kinetics of both sildenafil and tadalafil in rats, and the extent to which they cross the blood-brain barrier, have not been extensively studied. However, previously reported central effects in humans and rodents suggest that sildenafil (Schultheiss et al., 2002; Prickaerts et al., 2002; Brink et al., 2008) and tadalafil (Schiefer and Sparing 2005; Zhang et al., 2006a) effectively cross the blood-brain barrier, and the data from this study further support this.

In summary, the current project provides insight into the antidepressant-like properties of sildenafil in a genetic rat model of depression. Evidence for the involvement of PDE5 in its antidepressant-like response is provided, with a similar antidepressant-like profile demonstrated for tadalafil. Of note is that the interaction of sildenafil with the cholinergic system, as well as effects on behavioural correlates of serotonergic and

noradrenergic neurotransmission, appears to be dose-related. Although microdialysis studies are needed to further elucidate the involvement of these neurotransmitters in the antidepressant-like properties of the PDE5 inhibitors, the current data emphasise the dualistic properties of modulators of the NO-cGMP pathway in regulating depressive-like behaviour in rats, and provides insight into the specific neuronal pathways that may play a role. The potential of PDE5 as a novel target in the treatment of depression is underlined, and warrants more in-depth clinical study.

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FIGURE LEGENDS

Fig. 1 Inherent depressive-like behaviour of FSL rats compared to FRL controls as measured during a 5 min FST, and reversal of these effects following chronic fluoxetine treatment. (a) Immobility, (b) swimming and (c) climbing behaviours were recorded. A one way ANOVA followed by a Tukey-Kramer post-hoc analysis ($*** P < 0.001$) was performed for each behavioural parameter. The vehicle-treated FSL control group consisted of 24 rats ($n = 24$), whereas the remaining groups consisted of 12 rats each ($n = 12$).

Fig. 2 The dose-response relationship of the antidepressant-like properties of chronic sildenafil, with/without simultaneous mACh receptor blockade in FSL rats, as measured during a 5 minute FST. Effects on (a) immobility, (b) swimming and (c) climbing were recorded. A one way ANOVA followed by a Tukey-Kramer post-hoc analysis ($* P < 0.05$; $** P < 0.01$; $*** P < 0.001$) was performed for each behavioural parameter. The vehicle-treated control group consisted of 24 rats ($n = 24$), whereas the remaining treatment groups consisted of 12 - 18 rats each ($n = 12-18$).

Fig. 3 Behavioural antidepressant-like effects in FSL rats following chronic treatment with tadalafil with/without simultaneous mACh receptor blockade, as measured during a 5 min FST. Effects on (a) immobility, (b) swimming and (c) climbing were recorded. A one way ANOVA followed by a Tukey-Kramer post-hoc analysis ($* P < 0.05$; $*** P < 0.001$) was performed for each behavioural parameter. The vehicle-treated control group consisted of 24 rats ($n = 24$), whereas the remaining treatment groups consisted of 12 rats each ($n = 12$).

Fig. 1

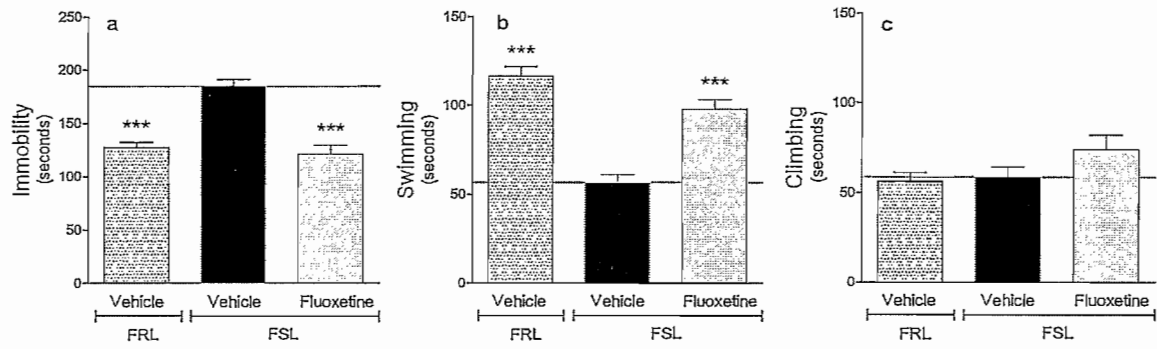


Fig. 2

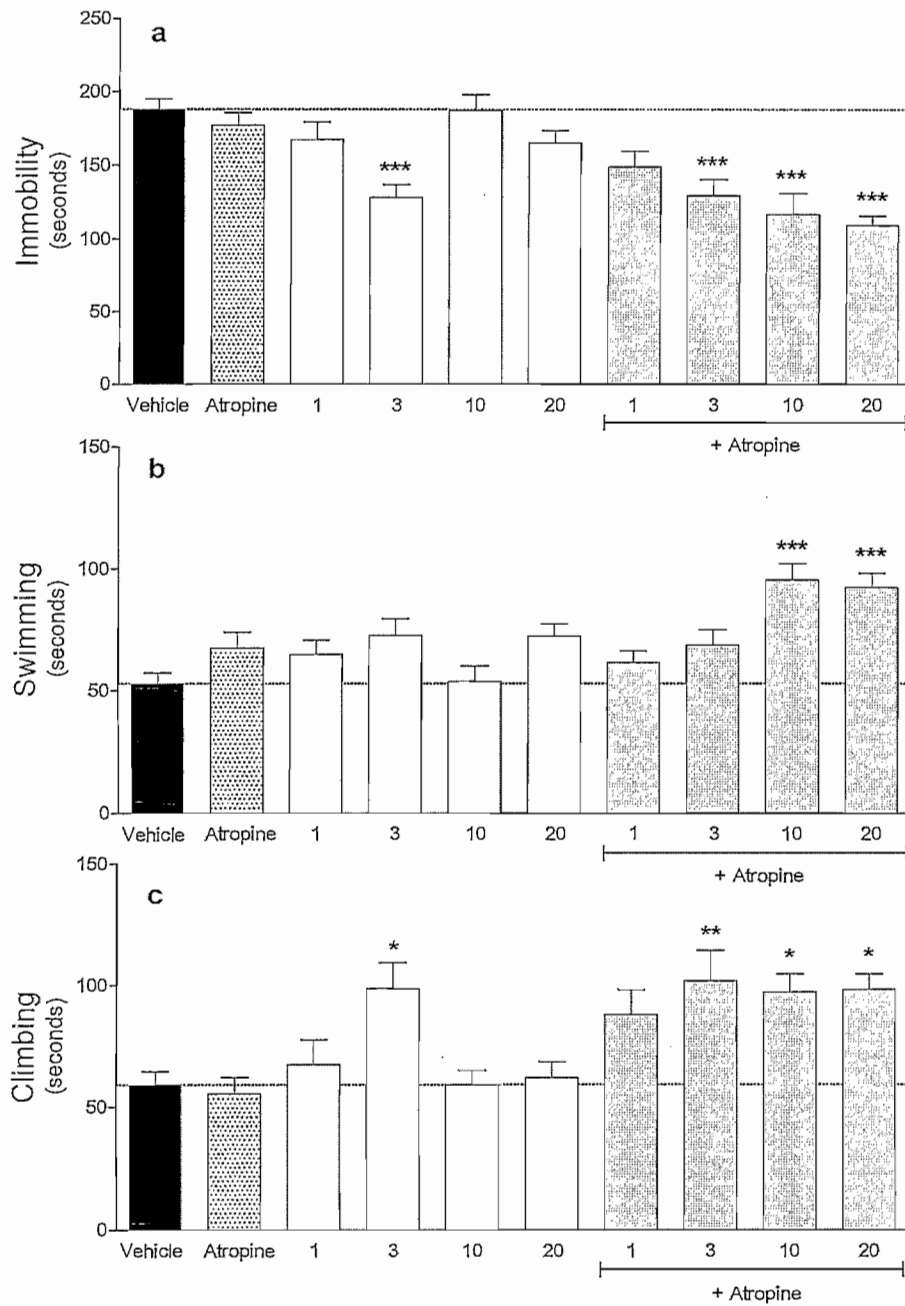
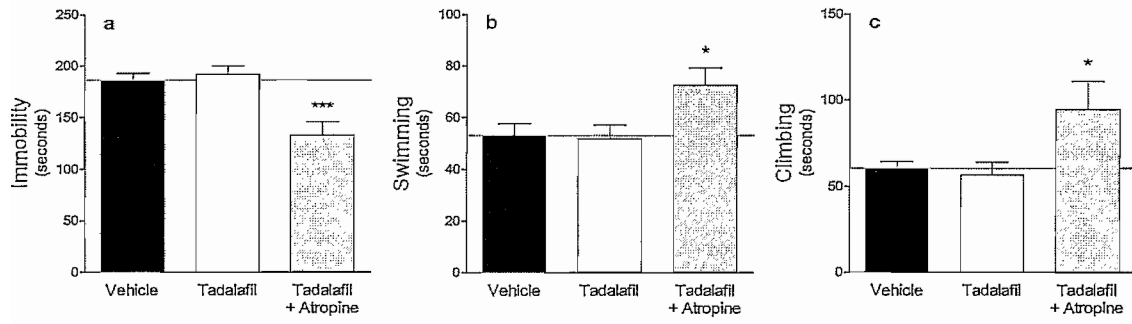


Fig. 3



Manuscript B

Chapter 4

In this chapter, a manuscript titled

“Investigating the role of protein kinase-G in the antidepressant-like response of sildenafil in combination with muscarinic acetylcholine receptor antagonism”

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

This manuscript has been provisionally accepted for publication on the precondition of some minor changes, and is presented here in its revised form.

TITLE PAGE**Title of paper**

Investigating the role of protein kinase-G in the antidepressant-like response of sildenafil in combination with muscarinic acetylcholine receptor antagonism.

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ABSTRACT

The cGMP/PK-G pathway plays a crucial role in neuroprotection and neurotrophin support, and is possibly involved in antidepressant action. Recently we reported on a novel antidepressant-like response following simultaneous administration of sildenafil (phosphodiesterase 5 (PDE5) inhibitor, thereby increasing cGMP levels), and atropine (muscarinic acetylcholine receptor antagonist) in the rat forced swim test (FST). However, it is unclear whether the antidepressant-like activity of sildenafil + atropine is mediated via the activation of PK-G, an important downstream effector for cGMP, and whether this may target known pathways in antidepressant action. We investigated whether the antidepressant-like response of sildenafil ± atropine could be reversed by Rp-8-Br-PET-cGMP, a PK-G inhibitor, and also whether a combination of 8-Br-cGMP (PK-G activator) ± atropine would likewise be active in the FST, and whether this combination could be attenuated by a PK-G inhibitor. 8-Br-cGMP alone, but not sildenafil alone, reduced immobility and selectively increased swimming in the FST. The antidepressant-like action of sildenafil was only evident following co-administration of atropine, and selectively increased climbing behaviour. Importantly, PK-G inhibition prevented the antidepressant-like effects of both 8-Br-cGMP and the sildenafil/atropine combination. These results confirm cholinergic-cGMP-PK-G interactions in the antidepressant-like effects of sildenafil, putatively acting via noradrenergic mechanisms, whereas direct PK-G activation induces antidepressant-like effects that are associated with enhancement of serotonergic neurotransmission.

Keywords: Phosphodiesterase 5, cGMP-dependent protein kinase, sildenafil, antidepressant, forced swim test, 8-Br-cGMP, atropine, cholinergic.

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INTRODUCTION

The pathogenesis of mood disorders remains elusive [16], while a significant proportion of depressed patients do not respond, or are only partial responders to current antidepressant treatment. Clinically used antidepressants are primarily modulators of monoamine neurotransmission, whereas more recent hypotheses regarding the neurobiological basis of depression and antidepressant action suggest that the disorder is multifactorial [17,27,28,30], and that dysfunctional monoamine neurotransmission alone is considered insufficient to fully explain all the dimensions of this disease. Theoretically, enhanced therapeutic outcome may be achieved by drugs that provide additional neuroprotective and neurotrophic support, thereby restoring the synaptic connectivity needed for normal neurotransmission [30].

The nitric oxide / cyclic guanosine 3'5'-monophosphate / protein kinase-G (NO/cGMP/PK-G) cell signalling pathway is believed to play a major role in neuroplasticity. Neuronal nitric oxide synthase (nNOS) is the enzyme responsible for nitric oxide (NO) production in neurons [13]. The physiological actions of NO are mainly mediated by stimulating soluble guanylate cyclase (sGC), which in turn leads to an increase in the synthesis of cGMP [13], which subsequently modulates the activity of cGMP-dependent PK-G, ion channels and phosphodiesterases (PDE) [12]. The rationale for investigating the potential of the NO/cGMP/PK-G pathway as a novel antidepressant strategy has multiple origins. The system plays a pivotal role in neuroplasticity [11,26,30] while it is also implicated in

neuroregulatory actions on neurotransmitter release [11,36]. Moreover, antidepressant-like effects for NOS and sGC inhibitors [9,10,21,24], including attenuation of such effects by drugs that increase cGMP levels (i.e. selective PDE5 inhibitors) [9,24] have been demonstrated in rodent models. Although the majority of these studies report antidepressant-like effects following inhibition of the NO/cGMP/PK-G pathway, there have been several studies demonstrating dual effects for NO donors and NOS inhibitors in rodent models of depression [8,22], suggesting that mood-regulation via the NO/cGMP/PK-G pathway is a highly complex process that is currently poorly understood.

Recently we reported on a novel antidepressant-like response following simultaneous modulation of the cGMP/PK-G and cholinergic systems. Co-administration of sildenafil, a selective PDE5 inhibitor (that would lead to increased cGMP levels), and atropine (a muscarinic acetylcholine receptor antagonist with central effects) was found to produce a robust antidepressant-like response in the rat forced swim test (FST), whereas both these drugs were devoid of any antidepressant-like effect when administered alone [2]. The rationale behind this combination was derived from observations that sildenafil increases cholinergic neurotransmission [2,33], an effect suggested to be depressogenic [23], while clinical studies have suggested that patients with depression present with deficits in NO signalling [1,6,32]. Furthermore, an interaction between cGMP and the cholinergic system is well recognised [36]. Consequently, increased cholinergic activity would therefore “mask” an underlying antidepressant

action for sildenafil, which can be revealed by concomitant anticholinergic treatment.

However, it is currently unclear whether the above-mentioned antidepressant-like effect of sildenafil + atropine, as observed in rodents, is directly dependent on PK-G activation following an elevation in cGMP levels. Therefore, in the current study we studied the antidepressant-like effects of the PK-G activator, 8-Br-cGMP, in the FST with or without concurrent central muscarinic receptor blockade, comparing these effects to that of sildenafil \pm atropine. Secondly, we investigated the modulatory action of the PK-G inhibitor, Rp-8-Br-PET-cGMP, on any anti-immobility effects produced by sildenafil + atropine and by 8-Br-cGMP \pm atropine. Thirdly, in order to confirm an association with existing mechanisms of antidepressant action, and since the NO-cGMP pathway can modulate monoaminergic transmission [36], we investigated the involvement of serotonergic and noradrenergic mechanisms in the effects of these drugs and combinations thereof in the FST, by respectively scoring swimming and climbing behaviour, as described by Cryan and colleagues [7].

MATERIALS AND METHODS

Animals. Male Sprague Dawley rats (Taconic MB A/S, Denmark) weighing 350 ± 15 g at the time of behavioural testing were housed 2 rats per cage at 20°C in a 12 h light /dark cycle (lights on at 7.00 a.m.). Tap water and food pellets were available ad libitum. The animals were allowed to acclimatise in the colony for 7 days before being used in experiments. All animal procedures were approved by the Danish National Committee for Ethics in Animal Experimentation (2007/561-1378).

Drugs and chemicals. Atropine (1 mg/kg, i.p.), imipramine (15 mg/kg, i.p.), 8-Br-cGMP (25 nmol, i.c.v.), Rp-8-Br-PET-cGMP (1 nmol, i.c.v.) and all chemicals used in the preparation of artificial cerebrospinal fluid (ACSF) and saline were purchased from Sigma (St. Louis, MO, U.S.A.). Sildenafil (10 mg/kg, i.p.) was donated by Pfizer (UK). The drug doses used in this study were based on a thorough literature survey [2,4,5,37,38,39].

Surgery. Under fentanyl-fluanisone (0.0945 and 0.3 mg/kg, respectively, i.m.) + midazolam (0.25 mg/kg, i.p.) anaesthesia, the rats were implanted with a custom made 25G guide cannula just above the right lateral ventricle at AP -1.0; L -1.6; DV -1.8 relative to bregma [34], and secured using three screws and dental acrylic. This positioning of the guide cannula allowed for intracerebroventricular (i.c.v.) infusion of drug and vehicle solutions via an injector tip (27G) entering the ventricle. The location of the injector tip was verified upon completion of the experiments by the infusion of a dye staining the injection site. Immediately after the operation, the animals were injected with buprenorphin (0.03 mg/kg, s.c.) and ampicillin (100

mg/kg, s.c.), and allowed to recover for 7 days before commencing with experiments. The rats were handled daily during the recovery period.

Experimental procedure. The modified forced swim test (FST) protocol was used as previously described [7]. On the first day of the experiment, rats were placed in acrylic plastic cylinders (60 cm in height, 24 cm in diameter) containing 40 cm of water (25°C) for 15 min. Immediately following the first swim session, the rats received the first of three injections of vehicle and/or drug solutions (i.p. and i.c.v.). The two remaining injections were given at 6 and 1 hours prior to the final swim test. Twenty four hours after the pre-swim session, the animals were reintroduced to the swim cylinders and their behaviour recorded digitally for 5 min. The rats were scored manually for displaying immobility, swimming or climbing behaviour.

In addition to the FST, and immediately prior to the final swim session, locomotor activity was recorded for 5 min in an open field arena (1 m²). The total distance travelled by each animal was scored from video using behavioural evaluation software (EthoVision XT® version 6.0.324, Noldus Information Technology, Waacheningen, The Netherlands). All experiments were carried out between 14:00 and 17:00.

Statistical analysis. Animals were randomly assigned to treatment groups according to a randomized block design and evaluated within a short time frame to minimise time-dependent variation. The data for immobility, swimming and climbing of all treatment groups were analysed using one-way analyses of variance (ANOVA) followed by Dunnett's multiple

comparison tests (GraphPad Prism® version 5.00, San Diego California, U.S.A.). All treatment groups were analysed simultaneously, although presented in separate figures to enhance clarity in the discussion. Data are expressed as the mean + S.E.M., and a value of $p < 0.05$ was considered to be statistically significant. The vehicle-treated control group consisted of 16 rats ($n = 16$) whereas the drug treatment groups consisted of 8-12 rats each ($n = 8-12$) (the specific number of animals in each group is given in Table 1).

RESULTS

As the positive control, imipramine was effective in significantly reducing immobility in the FST. Sildenafil in combination with atropine significantly reduced immobility in the FST relative to vehicle control, and comparable to the effect of imipramine, whereas both drugs were devoid of any effect when administered alone [$F(9, 109) = 4.69, p < 0.0001$; Fig. 1a]. Of note is that infusion of the PK-G activator, 8-Br-cGMP on its own significantly reduced immobility in the FST while this response was unaltered by the addition of atropine (Fig. 1a). After infusion of the PK-G inhibitor, Rp-8-Br-PET-cGMP, the anti-immobility effect of 8-Br-cGMP was no longer significantly different from vehicle control, whereas the significant antidepressant-like effect of sildenafil + atropine was now prevented, being partially attenuated by Rp-8-Br-PET-cGMP (Fig. 1b).

Alterations in swimming and climbing behaviour are shown in Fig. 2 and Fig. 3 respectively. Swimming was significantly increased by 8-Br-cGMP alone as well as the 8-Br-cGMP + atropine combination [$F(9, 109) = 3.29, p = 0.0014$; Fig. 2a]. This was in contrast to sildenafil + atropine, which selectively increased climbing behavior [$F(9, 109) = 3.55, p = 0.0007$; Fig. 3a]. Following simultaneous infusion of Rp-8-Br-PET-cGMP, the 8-Br-cGMP-induced increase in swimming behaviour was no longer significant relative to control (Fig. 2b), whereas Rp-8-Br-PET-cGMP attenuated the increase in climbing induced by sildenafil + atropine (Fig. 3b). Imipramine significantly increased climbing behaviour in the FST (Fig. 3a).

Lastly, locomotor activity was unaltered by all treatment conditions [$F(9, 109) = 1.12, p = 0.358$; data presented in Table 1], suggesting that changes in immobility in the FST did not result from alterations in motor activity.

DISCUSSION

An important result from the current study is that intraperitoneal administration of sildenafil plus atropine, and i.c.v. infusion of 8-Br-cGMP, an activator of PK-G, both produced an antidepressant-like response (reduced immobility) in the rat FST (Fig. 1a). Importantly, sildenafil alone was without effect. Furthermore, the anti-immobility effects of sildenafil plus atropine and that of 8-Br-cGMP were reversed by infusion of the PK-G inhibitor, Rp-8-Br-PET-cGMP (Fig. 1b), confirming the down-stream involvement of PK-G in both treatment responses. However, despite this common down-stream effect involving PK-G, the antidepressant-like profile of sildenafil, as demonstrated in the current study (Fig. 1a) and elsewhere [2], clearly involves a different up-stream mechanism to that of 8-Br-cGMP, since the antidepressant-like response of sildenafil could only be revealed with simultaneous inhibition of cholinergic neurotransmission (i.e. addition of atropine). This difference with respect to cholinergic involvement in the sildenafil response was also evident in the behavioural response in the FST, with 8-Br-cGMP increasing swimming (Fig. 2b) and sildenafil + atropine increasing climbing behaviour (Fig. 3b), suggesting serotonergic- and noradrenergic-mediated mechanisms, respectively. These observations suggest that the sildenafil-atropine combination mediates its antidepressant-like response primarily by bolstering noradrenergic

responses, whereas direct PK-G activation also evokes an antidepressant-like response, but via a serotonergic mechanism.

Although the pharmacological mechanisms underlying the antidepressant-like effects of the abovementioned treatments clearly involve the NO-cGMP pathway, their eventual antidepressant-like response can be related to modulation of noradrenergic and serotonergic neurotransmitter release, both of which are well recognised biological processes mediated by the cGMP/PK-G pathway [11,26,36]. The *in vivo* release of several neurotransmitters, including catecholamines, serotonin and acetylcholine, are generally increased in a variety of brain structures following local cGMP/PK-G stimulation, and includes the hippocampus, a brain region of importance in the neurobiology of depression [11]. In this context, central PDE5 inhibition following systemic sildenafil administration would lead to increased cGMP/PK-G functioning in different brain regions and neurons by virtue of elevated cGMP levels. PDE5 is expressed widely but differentially in various brain areas, and it has been shown to be expressed in cerebellum, caudate nucleus, hippocampus, substantia nigra as well as the subthalamic nucleus [29]. Therefore, inhibition of PDE5 may result in a wide range of effects, which may include enhancement of serotonergic, catecholaminergic and cholinergic neurotransmission. Indeed, it has been demonstrated that sildenafil has cholinomimetic effects [2,33], and if pro-cholinergic mechanism(s) are activated in certain brain areas such as the hippocampus, cortex [40] and basal forebrain [35],, this may result in a depressogenic response. Therefore, the antidepressant-like action of

sildenafil + atropine (Fig. 1a) may depend on uncoupling the provoked increase in cholinergic transmission, which would leave any enhanced noradrenergic transmission unattenuated.

An important finding in the current study is that, unlike sildenafil, 8-Br-cGMP decreases immobility in the FST in the absence of atropine (Fig. 1a). This result suggests that at the dose used in this study and following i.c.v. injection, 8-Br-cGMP induces a collective state of neuronal functioning predominately serotonergic in nature, which ultimately favours an antidepressant action. The response produced by 8-Br-cGMP was abolished by simultaneous infusion of the PK-G inhibitor, Rp-8-Br-PET-cGMP (Fig. 1b), which points to the PK-G dependency of this effect. On the other hand, this inhibitor only partially reversed the antidepressant-like response of sildenafil + atropine, suggesting some involvement for PK-G, but probably also a dependency on an additional down-stream mechanism of sildenafil. While this finding may reflect a potentially too low dose of the PK-G inhibitor (dose-response not evaluated), it may also emphasise the complexity of the physiological manifestations that may arise following PDE5 inhibition. For instance, elevated cGMP levels can provoke acetylcholine release [35] thereby setting up the observed cholinotropic response seen with sildenafil [2,33], whereas cGMP may also activate ion channels and alter the activity of several selective and non-selective phosphodiesterases, leading to altered cyclic nucleotide cross-talk between cGMP and cAMP [25]. Indeed, such a cholinergic response, leading to a "Yin-Yang" association between cyclic nucleotides, has been described

following lithium administration in rats [18,19]. This shift in sub-cellular second messenger function may also explain the more dominant noradrenergic-cAMP driven response seen with sildenafil (see below), especially when cholinergic activity is simultaneously inhibited, as opposed to the serotonergic-cGMP driven response evoked by 8-Br-cGMP. Indeed, NO-cGMP has been shown to target serotonergic signalling [3,31].

By distinguishing between swimming and climbing behaviour in the FST, an antidepressant-like effect may be characterised for the possible involvement of serotonergic and noradrenergic systems respectively [7]. As alluded to earlier, administration of 8-Br-cGMP selectively increases swimming behaviour (Fig. 2a), suggesting a serotonergic association for its antidepressant-like effect, and was prevented by infusion of a PK-G inhibitor (Fig. 2b). In agreement with this result, it has been suggested that the NO/cGMP/PK-G pathway has a mood-regulatory action that involves a serotonergic mechanism [15,20]. The NO precursor, *l*-arginine, enhanced the antidepressant-like effect of sertraline, a selective serotonin reuptake inhibitor [22], whereas low and ineffective doses of the NOS inhibitor, L-NAME, were able to potentiate the behavioural effects of imipramine and fluoxetine, but not reboxetine, a noradrenaline reuptake inhibitor, in the FST [14]. On the other hand, sildenafil + atropine selectively increases climbing behaviour (Fig. 3a), an effect which was partially attenuated by the infusion of a PK-G inhibitor (Fig. 3b), and suggests an antidepressant-like response associated with noradrenergic neurotransmission. Thus, although different mechanisms are at play, both treatment strategies display significant

antidepressant-like properties, emphasising the role of diverse nitrgic-cholinergic mechanisms in the antidepressant response. It should however, be noted that presynaptic modulation of transmitter release may not be the sole mechanism involved in the antidepressant responses following activation of the cGMP/PK-G pathway. Modulation of postsynaptic neuronal functioning, as well as regulatory actions on gene expression have been described for this system [26] and may also be involved in its mood-regulating function. That imipramine increases climbing (Fig. 3a) but not swimming behaviour (Fig. 2a) is somewhat unexpected when taking into account the relative selectivity of this drug for the serotonin transporter. This may, however, be explained by the fact that imipramine is metabolised to desipramine, a predominantly noradrenergic antidepressant.

In summary, the current study suggests that sildenafil presents with antidepressant-like effects driven partially through a PK-G and noradrenergic-mediated response that is dependent on suppression of cholinergic activity. Moreover, the study confirms that activation of the cGMP/PK-G pathway holds promise as a novel antidepressant target. Although further studies on other PDE5 inhibitors is needed, these data suggest that antidepressant mechanisms involving PDE5 inhibition or PK-G activation may involve complex neurobiological pathways that are not only dependent on cGMP signalling, but also on interactions with the cholinergic system. The involvement of the cGMP/PK-G system in mood regulation is

intricate, but provides an important way forward to understanding the neurobiology and treatment of depression.

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FIGURE LEGENDS

Fig. 1 (a) Anti-immobility responses in the FST of modulators of the cGMP/PK-G and cholinergic systems, and (b) the influence of a PK-G inhibitor on these effects. The following abbreviations were used: Veh (vehicle); Imipr (imipramine); Sild (sildenafil); Atr (atropine); 8-Br (8-Br-cGMP), Rp (Rp-8-Br-PET-cGMP). A one way ANOVA was performed for all groups, followed by a Dunnett (** $p < 0.01$) post-hoc analysis. The vehicle-control, sildenafil + atropine and 8-Br-cGMP treatment groups are shown in (a) and (b).

Fig. 2 (a) Effects on swimming behaviour in the FST by drugs that modulate the cGMP/PK-G and cholinergic systems, and (b) the influence of a PK-G inhibitor on these effects. The following abbreviations were used: Veh (vehicle); Imipr (imipramine); Sild (sildenafil); Atr (atropine); 8-Br (8-Br-cGMP). A one way ANOVA was performed for all groups, followed by a Dunnett (* $p < 0.05$) post-hoc analysis. The vehicle-control, sildenafil + atropine and 8-Br-cGMP treatment groups are shown in (a) and (b).

Fig. 3 (a) Effects on climbing behaviour in the FST by drugs that modulate the cGMP/PK-G and cholinergic systems, and (b) the influence of a PK-G inhibitor on these effects. The following abbreviations were used: Veh (vehicle); Imipr (imipramine); Sild (sildenafil); Atr (atropine); 8-Br (8-Br-cGMP); Rp (Rp-8-Br-PET-cGMP). A one way ANOVA was performed for all groups, followed by a Dunnett (* $p < 0.05$; ** $p < 0.01$) post-hoc analysis. The vehicle-control, sildenafil + atropine and 8-Br-cGMP treatment groups are shown in (a) and (b).

Fig. 1

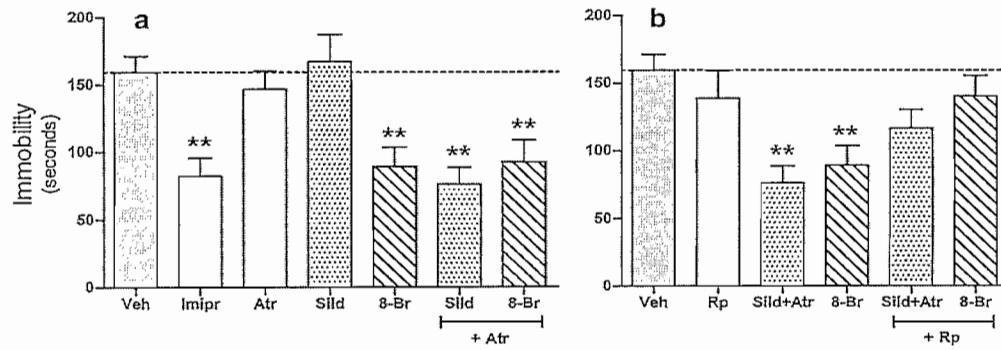


Fig. 2

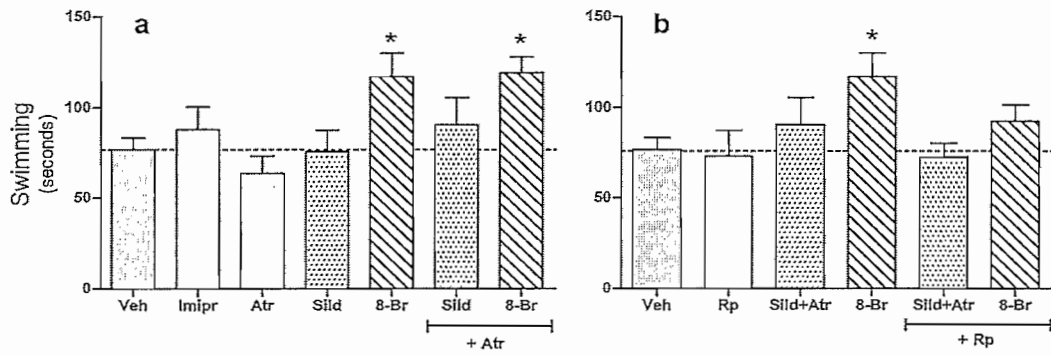


Fig. 3

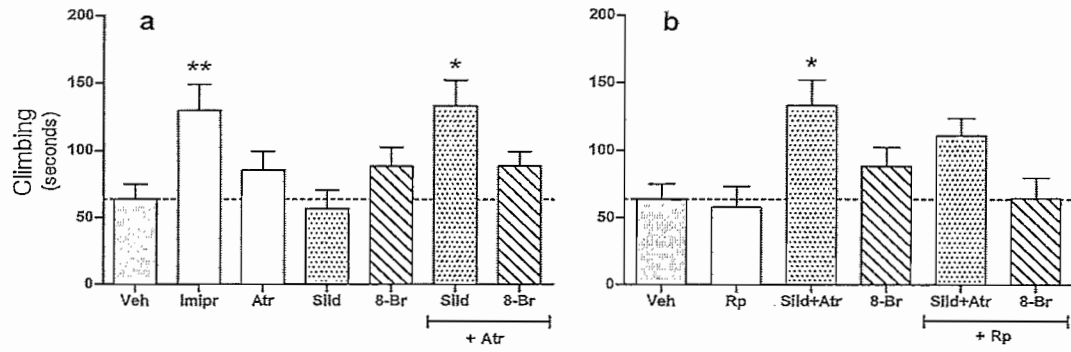


Table 1 Effects of cGMP/PK-G- or cholinergic modulators on locomotor activity

Treatment			Distance travelled (cm)
i.p.	i.c.v.	n-value	mean \pm S.E.M.
vehicle	vehicle	16	1851 \pm 270
imipramine (15 mg/kg)	vehicle	12	1621 \pm 280
sildenafil (10 mg/kg)	vehicle	10	1625 \pm 216
atropine (1 mg/kg)	vehicle	12	1547 \pm 172
vehicle	8-Br-cGMP (25 nmol)	12	1341 \pm 178
vehicle	Rp-8-Br-PET-cGMP (1 nmol)	11	1340 \pm 153
sildenafil + atropine	vehicle	8	2080 \pm 418
sildenafil + atropine	Rp-8-Br-PET-cGMP	12	1664 \pm 175
atropine	8-Br-cGMP	12	1662 \pm 251
vehicle	8-Br-cGMP + Rp-8-Br-PET-cGMP	8	1125 \pm 190

Manuscript C

Chapter 5

In this chapter, the manuscript titled

*“Decreased social interaction in Flinders Sensitive Line rats is increased
by chronic treatment with phosphodiesterase 5 inhibitors”*

is presented. This paper is ready to be submitted to **Behavioural Pharmacology** in the form of a short communication, and prepared according to the specific *Instructions to the Author* (provided in Addendum B). The references for this manuscript are provided at the end of this chapter.

TITLE PAGE

Title of article

Decreased social interaction in Flinders Sensitive Line rats is increased by chronic treatment with phosphodiesterase 5 inhibitors.

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ABSTRACT

The role of cGMP, or the response to PDE5 inhibitors, in anxiety is controversial. We evaluated the anxiolytic effect of chronic PDE5 inhibition in Flinders Sensitive Line (FSL) rats, a genetic animal model of depression that presents with increased anxiety-like behaviour. Rats were treated for 2 weeks with sildenafil (10 mg/kg) or tadalafil (10 mg/kg) or in combinations with atropine (1 mg/kg)), and evaluated in the social interaction test. Sildenafil and tadalafil, independent of atropine, increased social interaction in FSL rats, confirming an anxiolytic effect for chronic PDE5 inhibition.

Keywords: Flinders sensitive line rat, sildenafil, tadalafil, phosphodiesterase 5, anxiety, social interaction test, open field test, locomotor activity.

BODY TEXT

The nitric oxide (NO) – cyclic guanosine monophosphate (cGMP) pathway has been implicated in various processes in the central nervous system, including neuroplasticity (Manji et al., 2003; Serulle et al., 2008; Feil and Kleppisch 2008) and neuroprotection (Estevez et al., 1998; Nagai-Kusuhara et al., 2007), as well as in the regulation of neurotransmitter release (Prast and Philippu 2001; Feil and Kleppisch 2008). In addition, the NO–cGMP pathway has been shown to influence behaviours associated with anxiety (Eroglu and Caglayan 1997; Volke et al., 2003a; Volke et al., 2003b; Gilhotra and Dhingra 2009) and depression (Heiberg et al., 2002; Volke et al., 2003; Dhir and Kulkarni 2007) in rodents, and evidences for modulatory actions for NOS inhibitors (Vale et al., 1998; Monzon et al., 2001; Spiacci et al., 2008), precursors and donors of NO (Yildiz et al., 2000; Li and Quock 2002; Volke et al., 2003) and sGC inhibitors (Eroglu and Caglayan 1997; Kurt et al., 2004) have been reported. However, conflicting results have been obtained with regard to effects on anxiety-like behaviour. Whereas the majority of the results suggest that inhibition of the NO–cGMP pathway evokes anxiolytic effects (Volke et al., 1997; Yildiz et al., 2000; Volke et al., 2003; Gilhotra and Dhingra 2009) and that its stimulation is anxiogenic (Volke et al., 2003; Kurt et al., 2004; Gilhotra and Dhingra 2009), several others studies have shown that NO–cGMP inhibition may increase anxiety (Vale et al., 1998; Monzon et al., 2001), and also that activation of this pathway may be anxiolytic (Li and Quock 2002; Solis et al., 2008) in rodents. In addition, some studies report dual effects for drugs that modulate NO–cGMP activity in rodent models of anxiety and depression (Eroglu and Caglayan 1997; Spiacci et al., 2008).

Sildenafil and tadalafil are selective inhibitors of phosphodiesterase 5 (PDE5), the enzyme responsible for cGMP degradation, thereby leading to increased cGMP levels. Anxiogenic effects have been reported for sildenafil in mice (Volke et al., 2003; Kurt et

al., 2004; Gilhotra and Dhingra 2009), while no such studies have been performed on tadalafil. Importantly, while previous investigations into the anxiolytic or anxiogenic effects of sildenafil (Volke et al., 2003; Kurt et al., 2004; Gilhotra and Dhingra 2009) and other NO-cGMP modulators (Volke et al., 1997; Yildiz et al., 2000; Volke et al., 2003) have measured the acute effects of these drugs, a recent study reported an anxiolytic effect for sildenafil in the open field test, but following chronic (3 week) treatment (Solis et al., 2008). We also reported recently that sildenafil displays antidepressant-like activity in rats, but only following concurrent blockade of muscarinic acetylcholine (mACh) receptors with atropine (Brink et al., 2008). Given that sildenafil has inherent cholinotropic actions (Patil et al., 2004; Devan et al., 2004; Brink et al., 2008), and that increased cholinergic tone is associated with depression (Janowsky et al., 1972), we demonstrated that its antidepressant-like activity is revealed following uncoupling of an attenuating muscarinic drive. Recently, a similar observation was made for tadalafil, a structurally dissimilar PDE5 inhibitor (Liebenberg et al., 2009). In other words, it appears that activation of the NO-cGMP pathway by means of PDE5 inhibition is associated with a cholinergic interaction that may influence affective behaviour. Given that there is significant comorbidity between anxiety and depression (Hasler et al., 2004; Ressler and Mayberg 2007), and that several antidepressants are also effective in treating anxiety, a similar cGMP-cholinergic interaction in the control of anxiety-like behaviour following PDE5 inhibition is an interesting consideration.

The social interaction test is a widely used and accepted procedure for detecting anxiety-like behaviour in rodents. This test is sensitive to both anxiolytic and anxiogenic effects, and is sensitive to a number of environmental and physiological factors that can affect anxiety (File and Seth 2003). Flinders Sensitive Line (FSL) rats display inherent depression- and anxiety-like behaviours compared to their control strain, the Flinders Resistant Line (FRL) rats (Overstreet and Griebel 2004; Overstreet

et al., 2004). In addition to deficits in performance in the forced swim test, FSL rats spend significantly less time in social interaction compared to FRL controls (Overstreet and Griebel 2004; Overstreet et al., 2004). In the current study, we used this genetic model of increased depressive and anxiety-like behaviours to investigate any anxiolytic or anxiogenic effects that may result from chronic PDE5 inhibition by evaluating social interaction, as well as its possible dependence on manipulation of the cholinergic system.

We firstly validated the face and predictive validity of the animal model in our laboratory. One group of FRL rats (18 rats; $n = 9$) and a group of FSL rats (28 rats; $n = 14$) were treated with vehicle for 2 weeks, to demonstrate that FSL rats spend less time in social interaction than FRL controls (face validity). An additional group of FSL rats was treated for 2 weeks with fluoxetine (10 mg/kg, donated by Aspen, Port Elizabeth, South Africa) as a positive control (predictive validity). Secondly, we explored the effect of chronic PDE5 inhibition on social interaction in FSL rats, and the involvement of a possible PDE5-cholinergic interaction. Separate groups of FSL rats received chronic treatments with either sildenafil (10 mg/kg, a gift from Pfizer Global Research and Development, Kent, United Kingdom), tadalafil (10 mg/kg, kindly provided by Eli Lilly and Company, Indianapolis, IN, U.S.A.), or combinations of these drugs with atropine (1 mg/kg, Merck, Darmstadt, Germany). The drug doses were based on previous studies (Prickaerts et al., 2002; Overstreet and Griebel 2004; Zhang et al., 2006; Dawe et al., 2006; Brink et al., 2008). Rats were injected with vehicle (control) or vehicle plus drug(s) via daily intraperitoneal injections (injection volume = 0.5 ml) for 14 consecutive days. Rats weighed 300 ± 10 g on the day of behavioural testing, and were housed under standard conditions. All animal procedures were approved by the Ethics Committee of the North-West University (approval numbers: NWU-00009-07-A9

and NWU-00010-07-A0), and were in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

On the last day of treatment, the social interaction test was carried out as described previously for FSL rats (Overstreet and Griebel 2004). Approximately 12 hours following the final injection and 1 hour after the start of the dark cycle (19:00), rats were placed (in pairs) into a square test arena (1 m², marked with sixteen 25×25 cm blocks). Time spent in social interaction (grooming, licking sniffing, crawling over or under) as well as locomotor activity (number of line crossings) were recorded during a 5 minute session under low light, and scored by an observer who was blind to the treatment groups.

The data were analysed using one-way analyses of variance (ANOVA) followed by a Bonferroni's or Tukey-Kramer multiple comparison test (GraphPad Prism® version 5.00, San Diego California, U.S.A.). The data were pooled and analysed in the same groupings as presented in the figures. Results are expressed as the mean ± S.E.M., and a value of $p < 0.05$ was considered to be statistically significant. The n -values are given in the figure legends. As can be seen in Fig. 1, social interaction was significantly lower ($p < 0.05$) in vehicle-treated FSL rats compared to vehicle-treated FRL control rats [$F_{2,27} = 4.95$, $p = 0.0147$]. As the positive control, chronic treatment of FSL rats with fluoxetine significantly increased social interaction time compared to vehicle-treated FSL rats and did not differ to FRL control (Fig. 1). The results presented in Fig. 2 demonstrate that sildenafil and tadalafil with or without atropine significantly increased the time spent in social interaction in FSL rats compared to vehicle-treated FSL control [$F_{5,51} = 5.01$, $p = 0.0008$]. Atropine alone had no effect on social interaction in FSL rats, while it also did not influence the effect induced by sildenafil or tadalafil on social interaction time (Fig 2). Locomotor activity (number of line crossings) was significantly lower in vehicle treated FRL rats compared to vehicle

treated FSL rats [$F_{7,60} = 5.11$, $p = 0.0001$; Fig. 3]. None of the drug treatments significantly altered locomotor activity compared to vehicle control (Fig 3).

The use of FSL rats in the social interaction test has been validated in our laboratory for the evaluation of anxiety-like behaviour. We demonstrated that vehicle-treated FSL rats spend less time in social interaction than vehicle-treated FRL rats, and that chronic fluoxetine treatment restores social interaction in FSL rats to levels comparable to FRL controls (Fig. 1), providing face and predictive validity, respectively. Notably, chronic treatment with either sildenafil or tadalafil alone increased social interaction in FSL rats (Fig. 2) suggesting an anxiolytic effect for treatment with PDE5 inhibitors, and in the absence of atropine. This result concurs with a previous study demonstrating an anxiolytic action (measured as centre entries in the open field test) following chronic sildenafil administration (Solis et al., 2008). Moreover, these results also confirm that sildenafil and tadalafil have anxiolytic-like properties following chronic administration and that this response is common to structurally dissimilar PDE5 inhibitors. As mentioned above, acute dosing with sildenafil has anxiogenic effects (Volke et al., 2003; Kurt et al., 2004). Interestingly, serotonin reuptake inhibitors also increase anxiety after acute treatment, but downregulation of serotonin receptors after chronic treatment ultimately results in an anxiolytic response (Harvey 1997). Likewise, acute and chronic dosing with PDE5 inhibitors may also involve long term neuroregulatory effects that mediate anxiolytic effects over time.

Another important result from this study is that, unlike the antidepressant-like effect of PDE5 inhibitors that require co-administration of atropine (Brink et al., 2008; Liebenberg et al., 2009), the anxiolytic effects of sildenafil and tadalafil occur independently of muscarinic receptor inhibition. This suggests that, although the inhibition of PDE5 evokes both antidepressant-like and anxiolytic-like effects in rodent models, different mechanism(s) with regard to the cholinergic system are involved in

these behaviours responses. Finally, locomotor activity was unaltered for all treatments in this study. This indicates that alterations in social interaction are not secondary to locomotor effects (Fig. 3). FRL rats inherently displayed reduced locomotion compared to FSL rats. However, this does not explain increased social interaction in FRL rats (Fig. 1).

In conclusion, this study demonstrates anxiolytic effects following chronic PDE5 inhibition, by showing that sildenafil or tadalafil increases social interaction in FSL rats following 2 weeks of treatment in a pathological model for the measurement of anxiety-like behaviour.

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FIGURE LEGENDS

Fig. 1 Inherent anxiety-like behaviour in FSL rats compared to FRL controls, measured during a 5 minute social interaction test, and reversal of these effects following chronic fluoxetine treatment. A one way ANOVA followed by a Bonferroni's multiple comparison test was performed ($* p < 0.05$). The vehicle-treated FSL control group consisted of 28 rats ($n = 14$), whereas the remaining groups consisted of 14 - 18 rats each ($n = 7-9$).

Fig. 2 The effects of sildenafil and tadalafil (alone and in combination with atropine) on anxiety-like behaviour of FSL rats measured in terms of the time spent in social interaction during a 5 minute open field test. Abbreviations used in this figure: Veh (vehicle), Atr (atropine), Sild (sildenafil), Tada (tadalafil). A one way ANOVA followed by a Tukey-Kramer multiple comparison test was performed ($* p < 0.05$; $** p < 0.01$). The vehicle-treated FSL control group consisted of 28 rats ($n = 14$), whereas the remaining groups consisted of 12 - 18 rats each ($n = 6-9$).

Fig. 3 Inherent differences in locomotor activity between FSL and FRL rats, as well as the influence of different drug treatments on locomotor activity in FSL rats, measured in terms of the number of line crossings during a 5 minute open field test. Abbreviations used in this figure: Veh (vehicle), Flx (fluoxetine), Atr (atropine), Sild (sildenafil), Tada (tadalafil). A one way ANOVA followed by a Tukey-Kramer multiple comparison test was performed ($*** p < 0.001$). The vehicle-treated FSL control group consisted of 28 rats ($n = 14$), whereas the remaining groups consisted of 12 - 18 rats each ($n = 6-9$).

Fig. 1

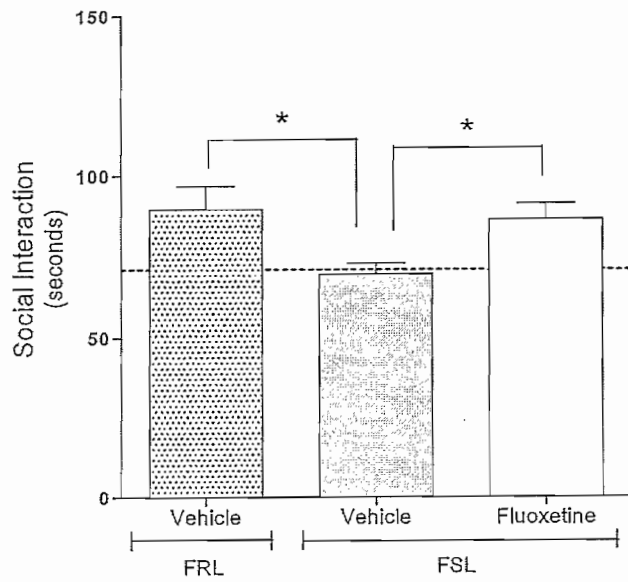


Fig. 2

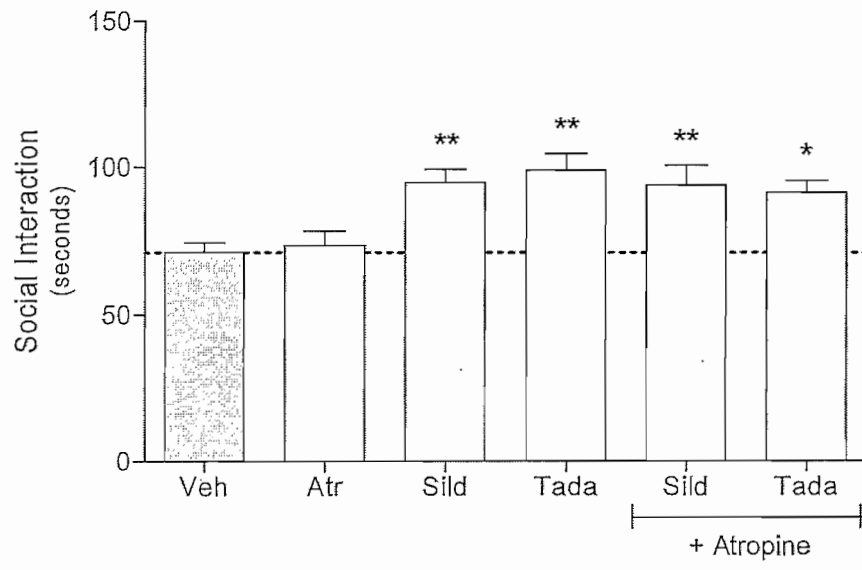
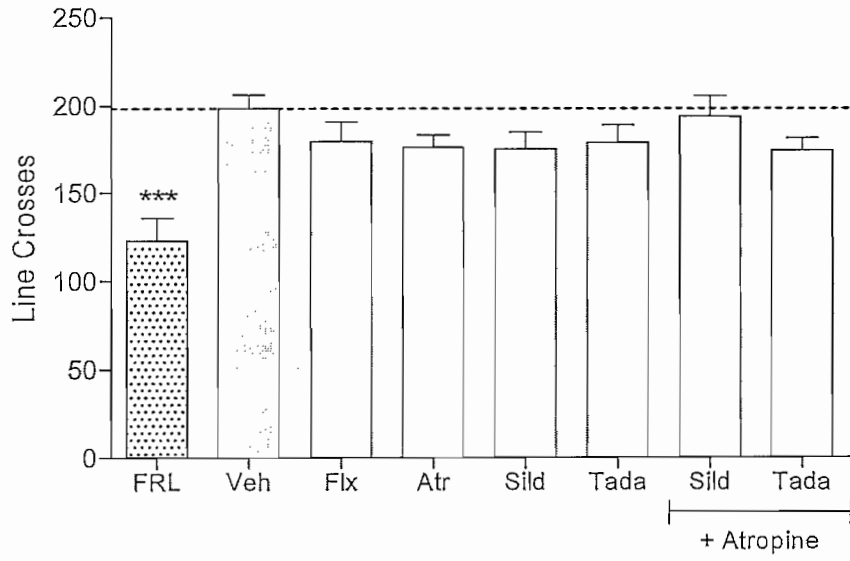


Fig. 3



Final Discussion

Chapter 6

In this chapter the results from the project as a whole will be summarised and the key findings discussed. Finally, the conclusions that can be drawn from this study will be discussed and suggestions of possible future experiments that may be carried out in follow-up studies provided.

6.1 Summary of results

Since the results of the current study are presented in separate sections, namely Chapters 3, 4 & 5 (scientific papers) and in Addendum A, a concise summary of all the results combined will be provided here. As outlined in Chapter 1, the experimental work in this project was performed in 3 separate phases, and the data will be provided accordingly.

6.1.1 Phase 1

The summarised results from this study are presented in Tables 6.1 – 6.3 below. We showed that chronic (14 days) treatment of FSL rats with sildenafil at doses of 3, 10 and 20 mg/kg significantly reduces immobility in the FST in combination with atropine (1 mg/kg), whereas sildenafil at 1 mg/kg did not affect immobility on its own or in combination with atropine (Chapter 3, Fig 2a) (see Table 6.1). Interestingly, sildenafil at a dose of 3 mg/kg reduced

Background

As described in Chapter 1, the primary objective of the current study was to investigate the antidepressant-like properties of the PDE5 inhibitors, sildenafil and tadalafil, in a genetic rodent model of depression (FSL rats), and formed the main focus of investigation for this phase of the project. In addition, interactions of sildenafil with other antidepressants, in terms of the augmentation of efficacy or acceleration of response, were also evaluated. Lastly, possible effects of sildenafil \pm atropine on the concentration of mACh receptors in the frontal cortex and hippocampus regions were assessed *ex vivo*. Rats were intraperitoneally with vehicle or test drugs for 7 or 14 days, whereafter assessment of locomotor activity and the FST were performed and the brain tissue analysed for mACh receptor concentration.

immobility in the absence of atropine, whereas the anti-immobility effects of sildenafil at doses of 10 and 20 mg/kg were reliant on simultaneous mACh receptor antagonism. Similarly, chronic treatment of FSL rats with tadalafil (10 mg/kg) + atropine decreased immobility in the FST, while tadalafil administration alone was without effect (Chapter 3, Fig. 3a). Climbing behaviour in the FST was increased following chronic treatment of FSL rats with sildenafil alone at a dose of 3 mg/kg, or with sildenafil at doses of 3, 10 and 20 mg/kg in combination with atropine (Chapter 3, Fig. 2c). Sildenafil increased swimming behaviour in FSL rats following chronic treatment at doses of 10 and 20 mg/kg in combination with atropine (Chapter 3, Fig. 2b). In similar fashion,

tadalafil (10 mg/kg) in combination with atropine significantly increased climbing (Chapter 3, Fig. 2c) and swimming (Chapter 3, Fig. 2b) behaviours in FSL rats the FST following chronic treatment, but not when administered alone.

Table 6.1 Behavioural effects of sildenafil and tadalafil (\pm atropine) in FSL rats

Treatment (dose)	14 days treatment			
	Forced swim test			Locomotor activity
	I	S	C	
Atropine (1 mg/kg)	↔	↔	↔	↔
Sildenafil (1 mg/kg)	↔	↔	↔	↔
(3 mg/kg)	↓	↔	↑	↔
(10 mg/kg)	↔	↔	↔	↔
(20 mg/kg)	↔	↔	↔	↔
Sildenafil (1 mg/kg) + Atropine	↔	↔	↔	↔
(3 mg/kg) + Atropine	↓	↔	↑	↔
(10 mg/kg) + Atropine	↓	↑	↑	↔
(20 mg/kg) + Atropine	↓	↑	↑	↔
Tadalafil (10 mg/kg)	↔	↔	↔	↔
(10 mg/kg) + Atropine	↓	↑	↑	↔

Note: In this table, an upwards arrow (↑) indicates an increase, a down arrow (↓) a decrease and a horizontal bidirectional arrow (↔) no difference in the indicated behavioural measurement relative to vehicle-treated FSL rats. Abbreviations used in this table: immobility (I), swimming, (S), and climbing (C).

Treatment of FSL rats with sildenafil (10 mg/kg) alone, or in combination with atropine, did not potentiate the antidepressant-like effect of fluoxetine (5 mg/kg) in the FST following chronic (14 days) treatment (Figure A.2a) (see Table 6.2). In addition, the antidepressant-like effect produced following chronic (14 days) treatment with sildenafil (10 mg/kg) in combination with imipramine (15 mg/kg) was not superior to the effect produced by imipramine alone (Figure A.2b). Treatment of FSL rats with fluoxetine for a shorter period (7 days) was insufficient for a significant antidepressant-like effect to be observed, and the addition of sildenafil (10 mg/kg) \pm atropine did not accelerate the onset of the antidepressant-like effect of fluoxetine (Figure A.3a). Administration of imipramine for this shorter treatment period produced a significant reduction in immobility in FSL rats in the FST on its own, whereas sildenafil did not potentiate the antidepressant-like effect of imipramine after 7 days of treatment (Figure A.3b).

Table 6.2 Interactions of sildenafil ± atropine with antidepressants in FSL rats

Treatment (dose)	7 days treatment		14 days treatment	
	Forced swim test	Locomotor activity	Forced swim test	Locomotor activity
	Immobility		Immobility	
Sildenafil (10 mg/kg)	↔	↔	↔	↔
Atropine (1 mg/kg)	↔	↔	↔	↔
Sildenafil + Atropine	↔	↔	↓	↔
Fluoxetine (5 mg/kg)	↔	↔	↓	↔
Fluoxetine + Sildenafil + Atropine	↔	↔	↓	↔
Imipramine (15 mg/kg)	↓	↓	↓	↓
Imipramine + Sildenafil	↓	↓	↓	↓

Note: In this table, an upwards arrow↑ indicates an increase, a down arrow (↓) a decrease and a horizontal bidirectional arrow (↔) no difference in the indicated behavioural measurement relative to vehicle-treated FSL rats.

Lastly, chronic (14 days) treatment of FSL rats with sildenafil alone or in combination with atropine did not significantly affect mACh receptor density in the frontal cortex or hippocampus regions of FSL rats (Figure A.7) (see Table 6.3)

Table 6.3 Effects of sildenafil ± atropine on mACh receptor density

Treatment (dose)	mACh receptor density	
	Frontal cortex	Hippocampus
Sildenafil (10 mg/kg)	↔	↔
Atropine (1 mg/kg)	↔	↔
Sildenafil + Atropine	↔	↔

Note: In this table, the horizontal bidirectional arrows(↔) indicate the absence of any effect on mACh receptor concentration in the indicated brain regions, compared to vehicle-treated FSL rats.

6.1.2 Phase 2

Sildenafil (10 mg/kg) + atropine (1 mg/kg) significantly reduced immobility, whereas both drugs were ineffective when administered alone (Chapter 4, Fig. 1a). Of note is that intracerebroventricular infusion of 8-Br-cGMP (25 nmol) significantly reduced immobility when administered alone or in combination with atropine. When Rp-8-Br-PET-cGMP (1 nmol, i.c.v.) was administered in combination with sildenafil + atropine or 8-Br-cGMP, the anti-immobility effects of these two treatments

Background

In this study we explored the mechanism(s) that may play a role in the antidepressant-like response induced by PDE5 inhibitors. More specifically, we investigated whether the activation of PK-G is involved in the antidepressant-like response evoked by sildenafil + atropine. Sprague-Dawley rats were treated subcutaneously (3 injections over a 24 hour period) with vehicle and/or drug solutions that were administered via intraperitoneal and intracerebroventricular injections (see Table 6.4). One hour following the final injection, the animals were subjected to the open field test

were no longer significant (Chapter 4, Fig. 1b). Moreover, sildenafil + atropine increased climbing behaviour in this paradigm (Chapter 4, Fig. 3a), while 8-Br-cGMP selectively increased swimming (Chapter 4, Fig. 2a). The simultaneous infusion of Rp-8-Br-PET-cGMP attenuated the increases in swimming (Chapter 4, Fig. 2b) and climbing behaviours (Chapter 4, Fig. 3b) induced respectively by 8-Br-cGMP and sildenafil + atropine in this paradigm.

6.1.3 Phase 3

Chronic treatment of FSL rats with sildenafil (10 mg/kg) or tadalafil (10 mg/kg) increased the social interaction time in FSL rats (Chapter 5, Fig. 2). These effects were unaltered by the co-administration of atropine. Locomotor activity was unaltered by all treatments.

Background

In this last phase of the project, we evaluated possible anxiolytic effects for inhibitors of PDE5 following chronic (14 days) treatment in rats that present with inherent anxiogenic-like behaviour (namely FSL rats), and investigated the involvement of the cholinergic system in this regard. Rats received daily intraperitoneal injections with vehicle or drug(s) for 14 days consecutive days (see Table 6.5), whereafter anxiety-like behaviour was assessed in the social interaction test

Table 6.4 Interactions in the FST by modulators of PK-G functioning

Treatment (dose)	2-day subacute treatment			
	Forced swim test			Locomotor activity
	I	S	C	
Imipramine (15 mg/kg, i.p.)	↓	↔	↑	↓
Atropine (1 mg/kg, i.p.)	↔	↔	↔	↔
Sildenafil (10 mg/kg, i.p.)	↔	↔	↔	↔
8-Br-cGMP (25 nmol, i.c.v)	↓	↑	↔	↔
Rp-8-Br-PET-cGMP (1 nmol, i.c.v)	↔	↔	↔	↔
Sildenafil + Atropine	↓	↔	↑	↔
8-Br-cGMP + Atropine	↓	↑	↔	↔
Sildenafil + Atropine + Rp-8-Br-PET-cGMP	↔	↔	↔	↔
8-Br-cGMP + Rp-8-Br-PET-cGMP	↔	↔	↔	↔

Note: In this table, an upwards arrow (↑) indicates an increase, a down arrow (↓) a decrease and a bidirectional arrow (↔) no difference in the indicated behavioural measurement relative to vehicle-treated FSL rats. Abbreviations used in this table: immobility (I), swimming, (S), and climbing (C).

Table 6.5 Effects of PDE5 inhibitors on anxiety-like behaviour

Treatment (dose)	14 days treatment	
	Social interaction	Locomotor activity
Atropine (1 mg/kg)	↔	↔
Sildenafil (10 mg/kg)	↑	↔
Sildenafil + Atropine	↑	↔
Tadalafil (10 mg/kg)	↑	↔
Tadalafil + Atropine	↑	↔

Note: In this table, an upwards arrow (↑) indicates an increase, a down arrow (↓) a decrease and a bidirectional arrow (↔) no effect on the indicated behavioural measurement relative to vehicle-treated FSL rats.

6.2 Discussion of key findings

PDE5 inhibitors display antimuscarinic-dependent antidepressant activity in FSL rats

In this project we confirm the previously reported antidepressant-like activity of sildenafil in combination with a mACh receptor antagonist (atropine) (Brink *et al.*, 2008), but here in a rodent genetic (chronic) model of depression, namely the FSL rats. Of note is that a similar antidepressant-like response is also demonstrated for tadalafil, a structurally unrelated PDE5 inhibitor and when co-administered with atropine, suggesting that an antidepressant-like action that is masked by a simultaneous increase in cholinergic tone may be a collective property of selective PDE5 inhibitors. This supports the notion that the inhibition of PDE5 may be an effective strategy for alleviating depression, and is in contrast to the current thinking that antidepressant-like effects may be induced by the inhibition of the NO/cGMP/PK-G pathway (as discussed in Chapter 2). In particular, previous studies have shown that sildenafil may in fact attenuate the antidepressant-like action of antidepressants (Kulkarni & Dhir, 2007; Dhir & Kulkarni, 2007; Jesse *et al.*, 2008; Ghasemi *et al.*, 2008). Although an inherent depressogenic effect following PDE5 inhibition was not evident in this study, or in the previous report from our group (Brink *et al.*, 2008), the results show that the uncovering of the antidepressant-like action of sildenafil and tadalafil is reliant on the simultaneous inhibition of mACh receptors. As mentioned before, it is believed that the potentiating effect that sildenafil (and possibly also tadalafil) has on the cholinergic system (Devan *et al.*, 2004; Patil *et al.*, 2004a; Brink *et al.*, 2008) may attenuate its antidepressant-like action, and may also explain the depressogenic-like action that has previously been reported for sildenafil in rats. The specific mechanism(s) by which this drug may potentiate cholinergic transmission remain unclear. However, the results from this study suggest that upregulation of mACh receptors in the hippocampus and frontal cortex regions is not involved in the pro-cholinergic action of sildenafil (Figure A.7). This said, the study by Brink *et al.* (2008) demonstrated an increase in metacholine-stimulated IP_x accumulation in human neuroblastoma cells that were pre-treated with sildenafil. Therefore, if receptor number is unaffected, potentiation of the downstream signalling cascade of mACh receptor functioning may well be involved in enhancing cholinergic transmission. Possible PK-G-mediated mechanisms include the phosphorylation of regulators of G-protein signalling (RGS proteins) and the regulation of the release of acetylcholine (as described in § 2.2.2.2). Furthermore, cGMP-induced alterations in cAMP signalling (cyclic nucleotide cross-talk) may also be involved in the cholinergic action of sildenafil.

Depressogenic cholinergic interactions are only stimulated at higher doses of sildenafil

Although a sildenafil dose of 1 mg/kg (alone or in combination with atropine) is insufficient for any antidepressant-like effects to be observed, a slightly higher dose (3 mg/kg) displays

antidepressant-like activity in the absence of atropine (Chapter 3, Fig 2a). However, doses higher than 3 mg/kg (i.e. 10 and 20 mg/kg) require co-administration of atropine for the antidepressant-like properties of sildenafil to be expressed. This suggests that the antidepressant- and depressogenic-like properties of sildenafil may be biphasic, with the antidepressant-like response initiated at a lower dose (albeit not necessarily a full response), whereas its depressogenic cholinergic effect is only activated at a higher dose. In like fashion, several other reports have demonstrated that NO donors and NOS inhibitors display dual effects in rodent models of depression (da Silva *et al.*, 2000; Inan *et al.*, 2004). With such a delicate dose-dependency of the resulting response, it may be concluded that all future studies investigating the antidepressant activity of PDE5 inhibitors or other modulators of the NO/cGMP/PK-G pathway, whether pre-clinical or clinical, should take into account these dose-response relationships. Such dualistic actions by modulators of the NO/cGMP/PK-G pathway may also explain the inconsistencies that currently exist in the data describing the role of this system in regulating mood and anxiety.

Sildenafil dose-dependently alters correlates of monoaminergic transmission in the FST

Not only does the dose of sildenafil determine its interaction with the cholinergic system in the FST, but its effects on behavioural correlates of monoaminergic neurotransmission also appear to be dose-related. The data from this study suggest that noradrenergic mechanisms may predominantly be activated by sildenafil at low doses, whereas higher doses of sildenafil (and tadalafil) also activate serotonergic and cholinergic mechanisms. Indeed, sildenafil exhibits actions similar to those of noradrenergic antidepressants on neuropathic pain (Jain *et al.*, 2001; Patil *et al.*, 2004a; Patil *et al.*, 2004b; Ambriz-Tututi *et al.*, 2005; Yoon *et al.*, 2009). Previous studies have demonstrated a biphasic effect for NO-donors on serotonin release, where enhanced serotonin release was associated with high doses of these drugs (Kaehler *et al.*, 1999). This is in line with the observation that sildenafil induces a serotonergic-like response exclusively at higher doses in the current study. Indeed, the modulation of neurotransmitter release may underlie some of the effects induced by sildenafil and tadalafil on noradrenergic- and serotonergic-associated behaviour in this study, especially since the release of both these monoamines have been shown to be NO-regulated (Prast & Philippu, 2001). However, elevated cGMP concentrations may also affect other targets (such as other types of PDEs) that may also play a role in the noradrenergic and serotonergic responses induced by the PDE5 inhibitors in this study (see below). Lastly, a serotonergic-like response was not induced by sildenafil (10 mg/kg) + atropine following subacute treatment, but only following chronic administration in FSL rats. This result may indicate that the serotonergic-like response of sildenafil (10 mg/kg) + atropine is dependent on adaptive changes that may occur following over time, and emphasises the importance of implementing chronic animal models in order to obtain a more complete understanding of the antidepressant profile of drugs.

The antidepressant action of sildenafil involves but is not limited to PK-G activation

The antidepressant-like response of sildenafil (10 mg/kg) + atropine is partially attenuated by the PK-G inhibitor, Rp-8-Br-PET-cGMP (1 nmol), whereas direct activation of this enzyme with 8-Br-cGMP (25 nmol) induces a significant antidepressant-like response. This suggests that the downstream activation of PK-G may well be involved in the antidepressant-like effects of PDE5 inhibitors. However, the antidepressant-like response of sildenafil + atropine in this study was not completely abolished by Rp-8-Br-PET-cGMP. As mentioned in Chapter 4, this may either indicate a too low dose for the PK-G inhibitor (the dose-response was not evaluated in this study) or that the antidepressant-like effects of sildenafil may also involve mechanism(s) other than the activation of PK-G. For instance, intracellular cGMP also regulates the activity of various selective- and non-selective PDEs. As noted in § 2.2.2.1.3., increased cGMP levels can stimulate cAMP signalling by inhibiting cAMP-selective PDE3, decrease the levels of both cGMP and cAMP by stimulating non-selective PDE2, or decrease its own concentration by stimulating PDE5 (Feil & Kleppisch, 2008). Sildenafil may therefore exert neuronal effects by not only activating PK-G-dependent targets, but also cAMP-stimulated effectors such as cAMP-dependent protein kinase (PK-A) and CREB. Increased cAMP signalling may also explain the noradrenergic-like responses of sildenafil observed in this study. Interestingly, the pro-cholinergic action of sildenafil also appear to rely on a mechanism other than PK-G activation, given that direct activation of PK-G with 8-Br-cGMP induces an antidepressant-like response in the absence of a mACh receptor antagonist in this study. However, it cannot be excluded that 8-Br-cGMP may also display a dose-dependent response profile with regard to cholinergic interactions in the FST, and this warrants further investigation.

Chronic treatment with PDE5 inhibitors induces anxiolytic-like effects in FSL rats

In this study we demonstrate that sildenafil and tadalafil display anxiolytic-like effects in the social interaction test by using rats that present with inherent anxiogenic-like behaviour, namely FSL rats. This finding is in contrast to the majority of previous pre-clinical reports that have suggested that inhibition of this pathway is anxiolytic (Volke *et al.*, 1997; Campbell *et al.*, 1999; Yildiz *et al.*, 2000; Volke *et al.*, 2003a), and that sildenafil has anxiogenic effects in mice (Volke *et al.*, 2003b; Kurt *et al.*, 2004). However, these studies have measured the acute actions of NO/cGMP/PK-G modulation, whereas this study suggests that repeated administration of PDE5 inhibitors may lead to adaptive changes that ultimately reduce anxiety. Indeed, a recent study has also reported anxiolytic-like effects in rats that were treated for 3 weeks with sildenafil (Solis *et al.*, 2008). In contrast to the antidepressant-like actions of sildenafil and tadalafil in this study, the anxiolytic responses of these drugs in the social interaction test do not involve interactions with the cholinergic system.

6.3 Conclusions

It is now well recognised that major depression is a psychiatric disorder with a neurobiological basis that is strongly linked to dysfunctional monoaminergic neurotransmission, as well as impaired neuroplasticity. Whereas several lines of evidence have indicated an important role for the Glu/NO/cGMP/PK-G signal transduction pathway in the defective neuroplasticity associated with depression, modulators of this pathway is currently only used as experimental antidepressants due to serious toxicity or side-effects. However, in the current study we have investigated a new target within the Glu/NO/cGMP/PK-G signal transduction pathway, namely the inhibition of PDE5, which may render novel antidepressants. In fact, drugs with this mechanism of action are already in clinical use, and PDE5 inhibitors may therefore hold the advantage of being tolerable in addition to having antidepressant activity. Furthermore, the current study has demonstrated that chronic PDE5 inhibition is anxiolytic, whereas other pre-clinical and clinical studies (pre-clinical and clinical) have demonstrated psychotropic actions for PDE5 inhibitors. These effects may contribute to the potential therapeutic applicability of PDE5 inhibitors as effective antidepressants.

This study has investigated the prospect of targeting PDE5 as a novel antidepressant strategy, and has produced several interesting outcomes. Not only have we now demonstrated antidepressant- and anxiolytic-like activity for PDE5 inhibitors (sildenafil and tadalafil) in a rat model with enhanced aetiological and construct validity, but we also provide insight into the antidepressant-like mechanism(s) that may be involved in the antidepressant-like action of these drugs. Although the responses of PDE5 inhibitors in the FST clearly involve an interaction with cholinergic neurotransmission, we demonstrate that this interaction may occur at a dose higher than that needed to induce an antidepressant-like response. Furthermore, the antidepressant-like mechanism(s) of PDE5 inhibitors appear to involve the activation of PK-G, although other mechanisms such as altered cyclic nucleotide cross-talk are also likely to play a role. Thus, the antidepressant-like action following PDE5 inhibition appears to be a multifaceted process that involves several depressogenic and antidepressant mechanisms that are dependent on the dose of the PDE5 inhibitor used, as well as on the duration of treatment, and may include possible modulating effects on cholinergic, noradrenergic and serotonergic neurotransmission.

Therefore, this study suggests that the key to utilising PDE5 inhibitors as clinically useful antidepressants may lie in utilising these drugs in a manner that leads to the selective expression of their antidepressant properties. This may be achieved, for instance, by the careful selection of the drug dose, or by the selective antagonism of their depressogenic mechanism(s). Furthermore, the enhancement of NO/cGMP/PK-G signalling by PDE5 inhibition may not only influence mechanisms involved in neuroplasticity, but also modulate

monoaminergic neurotransmission pathways that are implicated in depression, and hereby offer an antidepressant strategy that incorporates multiple hypotheses of the neurobiology of depression. Lastly, inhibitors of PDE5 may also hold a significant advantage over other modulators of the NO/cGMP/PK-G pathway in terms of tolerability and also possibly efficacy, since these drugs will only enhance the endogenous NO/cGMP/PK-G signal, as opposed to an artificial and ubiquitous enhancement of NO/cGMP/PK-G signalling by drugs that modulate the production of cGMP.

Finally, this study also emphasises the value of using animal models of depression not only as pre-clinical screening tools for the detection of antidepressant-like activity, but also to provide important indications of the neurochemical mechanisms that may be involved in the antidepressant-like responses produced. By using an animal model with enhanced aetiological and construct validity, the clinical human condition is more closely mimicked, thereby enhancing the probability for a more accurate prediction of responses and mechanisms that may have clinical relevance.

6.4 Recommendations and prospective studies

- Supportive behavioural evidence for the antidepressant-like properties of PDE5 inhibitors and activators of the NO/cGMP/PK-G pathway is warranted. Currently, the only evidence for antidepressant-like activity for these agents is from the FST, measuring behavioural despair. These drugs also need to be evaluated in behavioural tests that evaluate other depression- and anxiety-like behaviours as well as cognitive function, and may include tests such as the sucrose preference test (anhedonia), Y-maze (memory function), the novelty suppressed feeding test (stress-induced anxiety) or fear conditioning (hippocampus-dependent learning). Importantly, the dose-response relationships of NO/cGMP/PK-G modulators need to be considered in these tests, given the apparent dose-dependency of the mood-altering properties of these drugs. Interactions with the cholinergic system also need to be investigated in these experimental paradigms.
- Given the incomplete reversal of the anti-immobility effect of sildenafil + atropine by Rp-8-Br-PET-cGMP at the single dose used in this study, is still not clear whether the activation of PK-G is implicated in the downstream mechanism(s) of the antidepressant-like responses of PDE5 inhibitors. Therefore, a dose-response needs to be performed in terms of the abovementioned attenuating effect.
- The current study suggests that several neurotransmitters (i.e. acetylcholine, noradrenaline and serotonin) may be involved in the antidepressant-like actions of PDE5 inhibitors and direct activators of PK-G. Therefore, microdialysis studies are required to measure the extracellular concentrations of these neurotransmitters following acute and chronic treatment

with modulators of the NO/cGMP/PK-G system in brain regions associated with depression. The dose-dependency of the actions of these modulators on neurotransmitter release, as well as its dependency on simultaneous mACh receptor antagonism also warrants further investigation.

- The abovementioned effects on behaviour and neurotransmitter release need to be correlated with the function of the NO/cGMP/PK-G pathway, and the activity and/or expression of PK-G, PDE5, nNOS and soluble guanylyl cyclase, as well as the concentration of cGMP following acute or chronic administration of modulators of the NO/cGMP/PK-G signal transduction pathway.
- The effects of NO/cGMP/PK-G modulation on cAMP signalling in various brain areas may also provide valuable insight into possible cyclic nucleotide cross-talk that may be induced by these drugs, and how this may influence behavioural correlates of depression and cognition or neurotransmitter release. The concentration of cAMP in various brain regions, as well as activation or transcription of downstream effectors for cAMP such as PK-A and CREB may be measured.
- The actions of NO/cGMP/PK-G functioning on factors that are implicated in other hypotheses of depression also warrant further investigation. For instance, the dose-dependent actions of acute and chronic administration of modulators of this pathway on the functioning of the HPA-axis or the immune system may be investigated, as well as the interaction with the cholinergic system in this regard.
- The clinical evaluation of the antidepressant efficacy of the simultaneous administration of a PDE5 inhibitor and mACh receptor antagonist is an attractive prospect, especially since these drugs with these mechanisms of action are already approved for clinical use.

Addendum A: Additional Results

In this Addendum, additional results from experimental data that were not incorporated into the manuscripts (Chapters 3, 4 & 5) are provided, such as pilot studies and results that were not considered of a sufficient conclusive nature to be reported in a peer reviewed article. The experiments will be presented separately, with a short description of the aims, methods and results provided for each experiment, as well as a short discussion that indicates the significance of the data.

General methods for all experiments

Male FSL and FRL rats were used for the experiments in this study, weighing 300 ± 10 g on the day of testing. Rats 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 (approval numbers: NWU-00009-07-A9 and NWU-00010-07-A0), and were in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

A.1 Establish genetic difference between FSL and FRL rats

As mentioned in Chapter 2, the Flinders Sensitive Line (FSL) rat was originally developed in an attempt to create a strain of rats that is genetically more resistant to the organophosphate anticholinesterase agent, diisopropyl fluorophosphate (DFP), but the breeding program led to the development of a line of rats that was more sensitive to the effects of DFP (Russell & Overstreet, 1987). It was later observed that these rats also present with specific biological and behavioural differences that are associated with human depression, and this model has subsequently been validated as a genetic rat model of depression (Overstreet *et al.*, 2005a). In addition, FSL rats are more sensitive to serotonergic agonists and display an exaggerated hypothermic response to the 5-HT_{1A} receptor agonist, 8-OH-DPAT, relative to FRL rats (Overstreet *et al.*, 1994). The difference in response to the effect of this drug can therefore be used to distinguish between FSL and FRL rats, and to confirm the genetic distinction between these strains.

Aims

The FSL and FRL rat colonies in our laboratory were recently obtained from a distant source, and have not been used in experiments prior to this study. Therefore, we needed to confirm the genetic dissimilarity between our FSL and FRL colonies before any experiments could be initiated. The increased sensitivity of FSL rats to the hypothermic effect of 8-OH-DPAT was used to differentiate between the strains.

Methods

One group FSL ($n = 10$) and one group FRL rats ($n = 10$) were randomly selected from the colonies in our laboratory. Baseline core body temperatures were obtained, whereafter rats were injected (i.p.) with 8-OH-PAT (0.1 mg/kg, 0.9% saline as vehicle). After 30 minutes, a second body temperature measurement was obtained, and the temperature difference relative to baseline calculated for each rat. Data was analysed using a student- t test and a value of $p < 0.05$ was considered to be statistically significant.

Results

As illustrated in Figure A.1, FSL rats presented with an increased sensitivity to the hypothermic effect of 8-OH-DPAT ($p = 0.0037$).

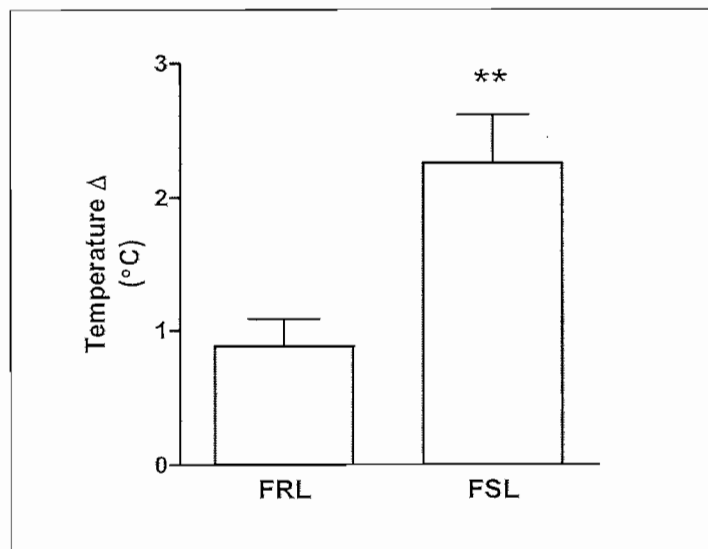


Figure A.1 The difference in the hypothermic responses of FSL and FRL rats as measured 30 minutes after injection with 8-OH-DPAT. The data was analysed using a student- t test and is expressed as the mean + SEM (** $p < 0.01$). Both groups consisted of 10 rats each ($n = 10$).

Discussion

This experiment confirms the supersensitivity of FSL relative to FRL rats to the effect of a serotonergic agonist. The genetic difference between the FSL and FRL colonies was now confirmed, allowing further experimentation with FSL rats in our laboratory.

A.2 Interactions of sildenafil with antidepressants in FSL rats

When taking into account the promising data suggesting an antidepressant-like action for sildenafil + atropine, augmentation (in efficacy and onset of action) of two distinct classes of known antidepressants (viz. fluoxetine and imipramine) with this combination appears to be a promising prospect. We investigated possible interactions of sildenafil + atropine and fluoxetine, as well as of sildenafil and imipramine (an antidepressant with inherent antimuscarinic activity) in FSL rats and by measuring immobility in the FST as a parameter of antidepressant-like action. Locomotor activity was measured as a control measure to detect possible increases in locomotor activity induced by the treatments that may influence performance in the FST. The locomotor data for the rats used in this objective (interactions of sildenafil with antidepressants) is presented here, as well as that of the experiments in Chapter 3 that were not included in the manuscript.

Behavioural testing protocols for experiments in A.2.1 – A.2.3:

Approximately 12 hours following the final injection, and 1 hour after the start of the dark cycle (19:00), the rats were subjected sequentially to the open field test and 2 hours thereafter, to the FST.

Open field test: The open field test was carried out as previously described (Overstreet & Griebel, 2004). Rats were placed into a square test arena (1 m², marked with sixteen 25 × 25 cm blocks), and their behaviour recorded on video for 5 minutes. The total number of line crossings was scored as a measure of locomotor activity.

Forced swim test: The forced swim test was performed as previously described for FSL rats (Dremencov *et al.*, 2004; Overstreet & Griebel, 2004). Rats were placed into plexiglass cylindrical tanks (40 cm high and 18 cm in diameter) containing 18 cm of water maintained at 23°C, and behaviour recorded on video under red light illumination for 5 minutes. Rats were scored as immobile when only the necessary movements were made to keep their heads above the water.

A.2.1 Augmentation strategies

Aims

We investigated whether sildenafil + atropine may potentiate the antidepressant-like response of fluoxetine in FSL rats in the FST, and/or whether sildenafil may act synergistically with imipramine in this rat model, given the inherent antimuscarinic activity of this antidepressant.

Methods

Separate groups of FSL rats were injected daily (i.p.) with vehicle (5% DMSO in saline), fluoxetine (5 mg/kg), imipramine, (15 mg/kg), sildenafil (10 mg/kg) or atropine (1 mg/kg), as well as combinations of fluoxetine and imipramine with sildenafil ± atropine for 14 consecutive days. Behavioural testing was carried out as described above. The data were analysed using a one-way ANOVA, followed by a Tukey-Kramer multiple comparison test, and a value of $p < 0.05$ was considered statistically significant. The number of rats in each group (n -values) is given in the figure legend (Figure A.2).

Results

Chronic treatment with fluoxetine or imipramine alone significantly decreased immobility time in FSL rats [$F(7,99) = 10.65$; $p < 0.0001$; Figure A.2]. However, sildenafil + atropine did not potentiate the antidepressant-like response of fluoxetine, whereas the response produced by imipramine was not significantly altered by the addition of sildenafil.

Discussion

These results suggest that sildenafil ± atropine may not act synergistically with fluoxetine or imipramine in this model of depression. However, it cannot be excluded that the maximum measurable antidepressant-like response for this animal model may have been reached with these drugs, thereby preventing the detection of any synergistic effects by sildenafil ± atropine.

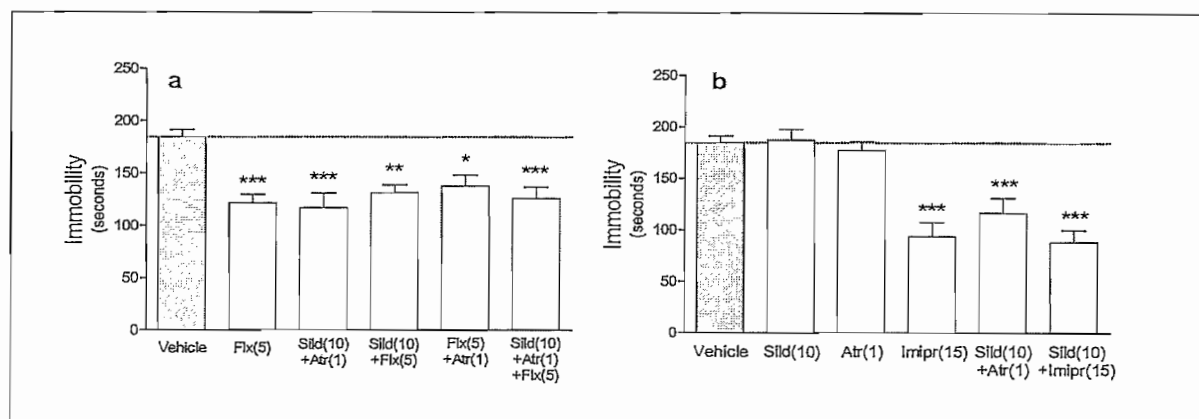


Figure A.2 Antidepressant-like effects in the FST following chronic (14 day) treatment with (a) fluoxetine or (b) imipramine, and combinations of these antidepressants with sildenafil \pm atropine in FSL rats, measured in terms of immobility time during a 5 minute test session. Data from (a) and (b) were analysed collectively using a one-way ANOVA, followed by a Tukey-Kramer multiple comparison test, and are expressed as the mean + SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). All groups consisted of 10-12 rats each ($n = 10-12$), except for the vehicle control group which consisted of 24 rats ($n = 24$). The following abbreviations are used: Flx (fluoxetine); Imipr (imipramine); Sild (sildenafil); Atr (atropine).

A.2.2 Evaluation of hastening of onset of action

A key characteristic of the FSL rat as a model of depression is that chronic antidepressant treatment is required to normalise the exaggerated immobility displayed by FSL rats in the FST when using a previously described protocol for this test (Overstreet, 1993; Overstreet *et al.*, 1995a; Dremencov *et al.*, 2004). As this more accurately resembles the clinical scenario in humans, the model is rendered suitable for the pre-clinical evaluation of the onset of action of antidepressant drugs (Dremencov *et al.*, 2004).

Aims

In this experiment, we investigated whether sildenafil \pm atropine may accelerate the onset of antidepressant-like action of fluoxetine or imipramine in FSL rats in the FST.

Methods

Separate groups of FSL rats were injected daily (i.p.) with vehicle (5% DMSO in saline), fluoxetine (5 mg/kg), imipramine, (15 mg/kg), sildenafil (10 mg/kg) or atropine (1 mg/kg), as well as combinations of fluoxetine and imipramine with sildenafil \pm atropine for a shorter, 7 day treatment period. Behavioural tests were carried out as described in collective protocol above. The data were analysed using a one-way ANOVA, followed by a Tukey-Kramer multiple comparison test, and a value of $p < 0.05$ was considered statistically significant. The number of rats in each group (n -values) is given in the figure legend (Figure A.3).

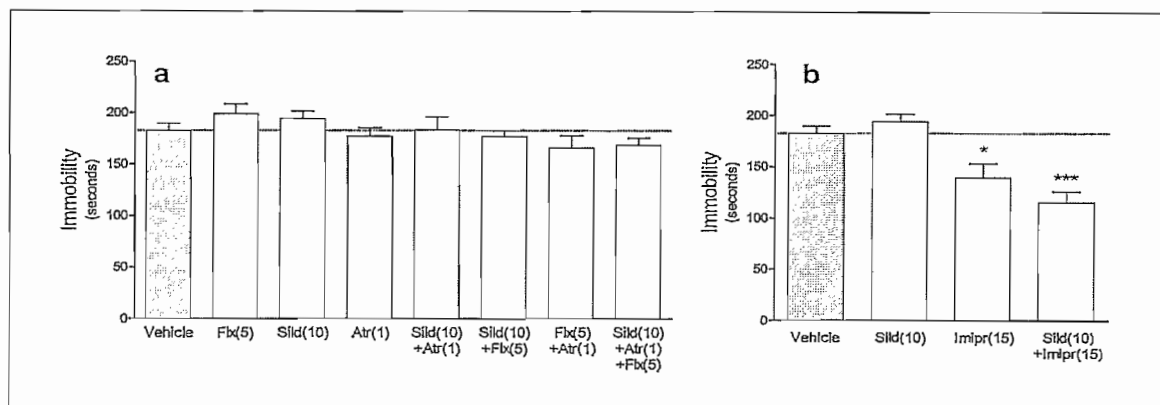


Figure A.3 Antidepressant-like effects in the FST following a shorter (7 day) treatment period with (a) fluoxetine or (b) imipramine, and combinations of these antidepressants with sildenafil \pm atropine in FSL rats, measured in terms of immobility time during a 5 minute test session. Data from (a) and (b) were analysed collectively using a one-way ANOVA, followed by a Tukey-Kramer multiple comparison test, and are expressed as the mean + SEM (* $p < 0.05$; *** $p < 0.001$). All groups consisted of 10-12 rats each ($n = 10-12$), except for the vehicle control group which consisted of 22 rats ($n = 22$). The following abbreviations are used: Flx (fluoxetine); Imipr (imipramine); Sild (sildenafil); Atr (atropine).

Results

As shown in Figure A.3a, fluoxetine alone, sildenafil + atropine, or the combination of these treatments did not reduce immobility in the FST after a shorter (7 day) treatment period [$F(9,122) = 6.74$; $p < 0.0001$]. However, imipramine alone significantly reduced immobility after 7 days (Figure A.3b). The response evoked by imipramine + sildenafil was not significantly different from the response produced by imipramine alone or sildenafil + atropine.

Discussion

That fluoxetine and sildenafil + atropine failed to evoke a significant antidepressant-like response after a shorter treatment period (Fig. A.3a) is in line with the validity of this model as a chronic model for depression. However, sildenafil + atropine does not shorten the onset time for fluoxetine in this rat model, suggesting that simultaneous inhibition of PDE5 and cholinergic neurotransmission, and selective inhibition of serotonin reuptake, does induce a more rapid onset of antidepressant-like action. That imipramine treatment for 7 days induces a significant antidepressant-like response in FSL rats in the FST (Figure A.3b) is most likely a result of a too high dose in this study. To more effectively investigate a possible hastening of the onset of antidepressant-like action of imipramine by sildenafil, experiments with sub-effective doses of imipramine are needed. However, the result in this study does not exclude the possibility that sildenafil and imipramine may act synergistically in this regard.

A.2.3 Measurement of locomotor activity

As mentioned above, locomotor activity was assessed as a rule before subjecting the rats to the FST. In this section, locomotor activity data for the rats used in the augmentation strategies described above (§ A.2.1 and A.2.2) will be presented, as well as the locomotor data for the experiments described in Chapter 3 that were not included in the manuscript.

Aims

We evaluated possible alterations in locomotor activity following all treatments in this study to eliminate the possibility of false positives in the FST. That is, we screened for possible induced increases in locomotor activity that may affect performance in the FST and lead to incorrect assumptions.

Methods

A group of FRL rats were injected daily (i.p.) with vehicle (5% DMSO in saline) for 14 days. Separate groups of FSL rats were injected daily (i.p.) with vehicle, fluoxetine (5 mg/kg), imipramine, (15 mg/kg), sildenafil (1, 3, 10 and 20 mg/kg) or atropine (1 mg/kg), as well as combinations of fluoxetine and imipramine with sildenafil \pm atropine for 7 or 14 days. Additional groups of FSL rats were also treated with tadalafil (10 mg/kg) or tadalafil + atropine (1mg/kg). Behavioural testing was carried out as described in the protocol above. The data was analysed using one-way ANOVA, followed by Tukey-Kramer multiple comparison tests, and a value of $p < 0.05$ was considered statistically significant. The number of rats in each group (n -values) is given in the figure legends (Figure A.4, A.5 and A6).

Results

Vehicle-treated FRL rats displayed significantly reduced locomotor activity compared to vehicle-treated FSL rats [$F(2,45) = 12.0$; $p < 0.0001$; Figure A.4a]. Fluoxetine treatment did not alter locomotor activity in FSL rats. Similarly, sildenafil did not alter locomotor activity in FSL rats at any of the doses used in this study, alone or in combination with atropine [$F(9,122) = 1.52$; $p = 0.15$; Figure A.4b]. There were also no significant changes in locomotor activity of FSL rats following treatment with tadalafil alone or in combination with atropine [$F(2,40) = 1.69$; $p = 0.20$; Figure A.4c]. As shown in Figure A.5, locomotor activity was also unaltered in FSL rats treated for 14 days with fluoxetine alone or in combination with sildenafil \pm atropine. However, imipramine significantly decreased locomotor activity alone, and in combination with sildenafil [$F(9,108) = 19.3$; $p < 0.0001$]. Figure A.6 shows that that 7 days treatment of FSL rats with fluoxetine alone or in combination with sildenafil \pm atropine, did not affect locomotor activity. However, 7 days of treatment with imipramine

alone or imipramine + sildenafil significantly decreased locomotor activity in FSL rats in the open field test [$F(9,103) = 10.7$; $p < 0.0001$].

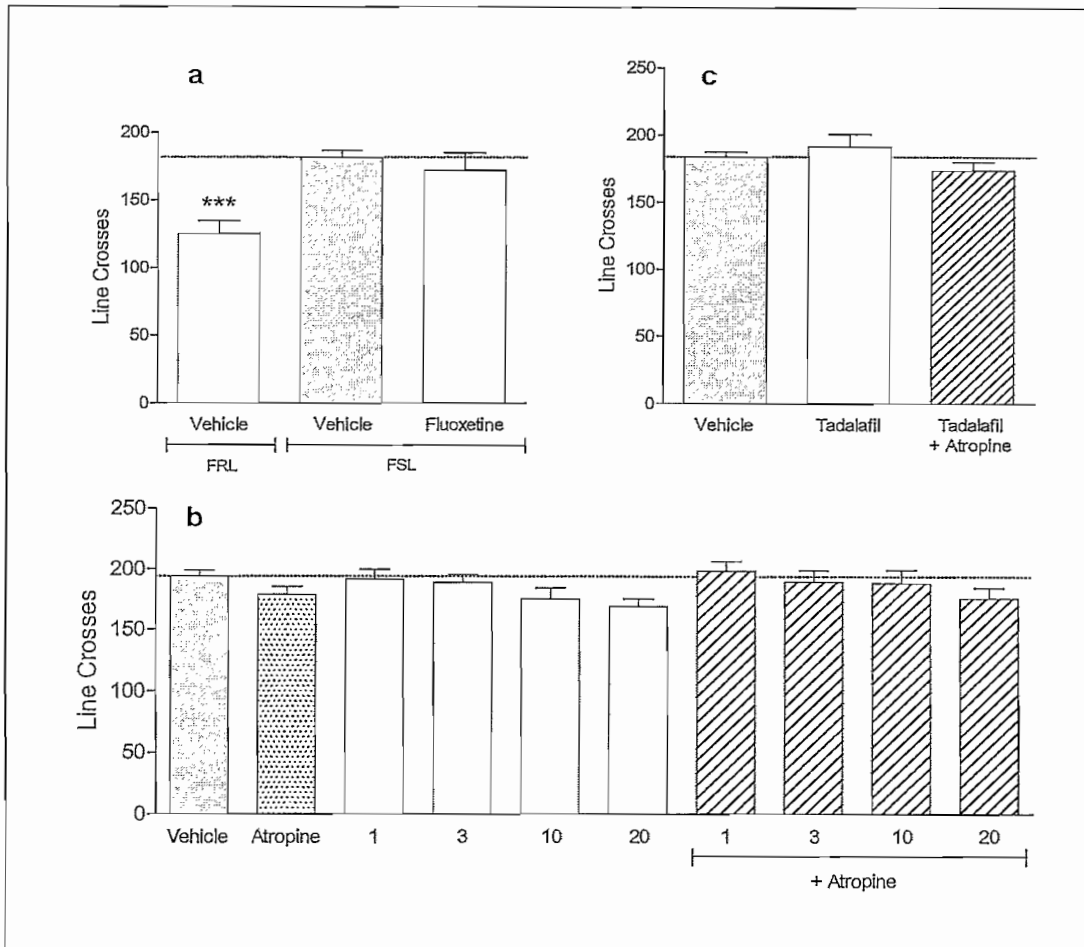


Figure A.4 Locomotor activity of (a) vehicle-treated FRL and FSL rats and FSL rats treated with fluoxetine, (b) FSL rats treated with different doses of sildenafil alone or in combination with atropine, and (c) FSL rats treated with tadalafil alone or in combination with atropine, measured as the number of line crosses counted during a 5 minute open field test, following 14 days of treatment. Data for each graph was analysed separately using a one-way ANOVA, followed by a Tukey-Kramer post-hoc analysis, and are expressed as the mean + SEM (** $p < 0.001$). Separate vehicle control groups were used for each analysis. The FSL vehicle-treated control groups consisted of 24 rats each ($n = 24$), whereas all other groups consisted of 12-18 rats each ($n = 12-18$).

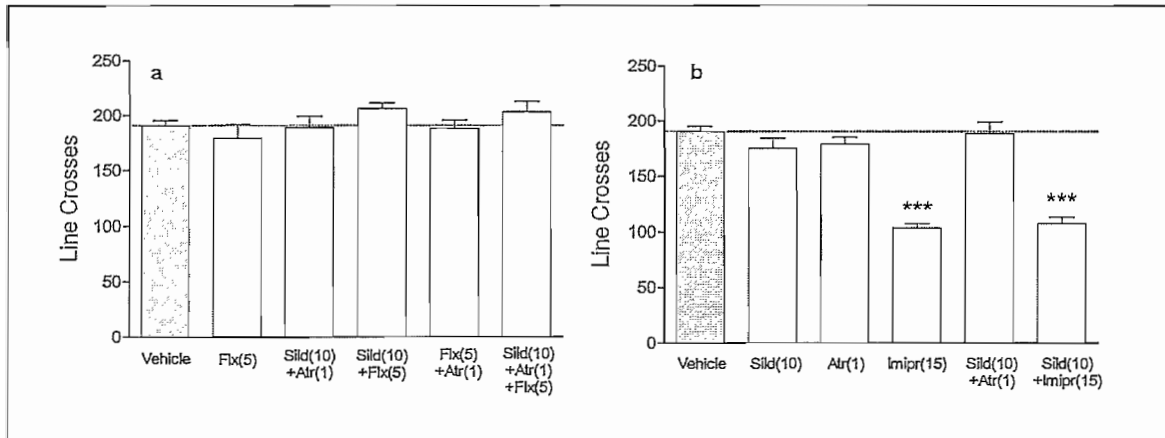


Figure A.5 Locomotor activity measured following chronic (14 day) treatment with (a) fluoxetine or (b) imipramine, and combinations of these antidepressants with sildenafil \pm atropine in FSL rats, in terms of the number of line crosses during a 5 minute open field test. The data in (a) and (b) were analysed collectively using a one-way ANOVA, followed by a Tukey-Kramer post-hoc analysis and are expressed as the mean \pm SEM. (***) $p < 0.001$. All groups consisted of 10-12 rats each ($n = 10-12$), except for the vehicle control group which consisted of 24 rats ($n = 24$). The following abbreviations are used: Flx (fluoxetine); Imipr (imipramine); Sild (sildenafil); Atr (atropine).

Discussion

In contrast to previous studies have demonstrated increased locomotor activity in FRL rats relative to FSL rats (Overstreet *et al.*, 2004; Overstreet & Griebel, 2004), this study demonstrated lower locomotor activity in FRL rats compared to FSL rats (Figure A.4a). Nevertheless, this result can not account for the increased mobility of FRL rats measured in the FST. In addition, the reduction in locomotor activity induced by imipramine after 7 and 14 days of treatment in FSL rats can also not explain the decreases in immobility in the FST following these treatment regimes. None of the other drugs or drug combinations in this study altered locomotor activity compared to vehicle-treated FSL control. Therefore, antidepressant-like effects observed for these treatments in the FST are not secondary to increases in locomotor activity.

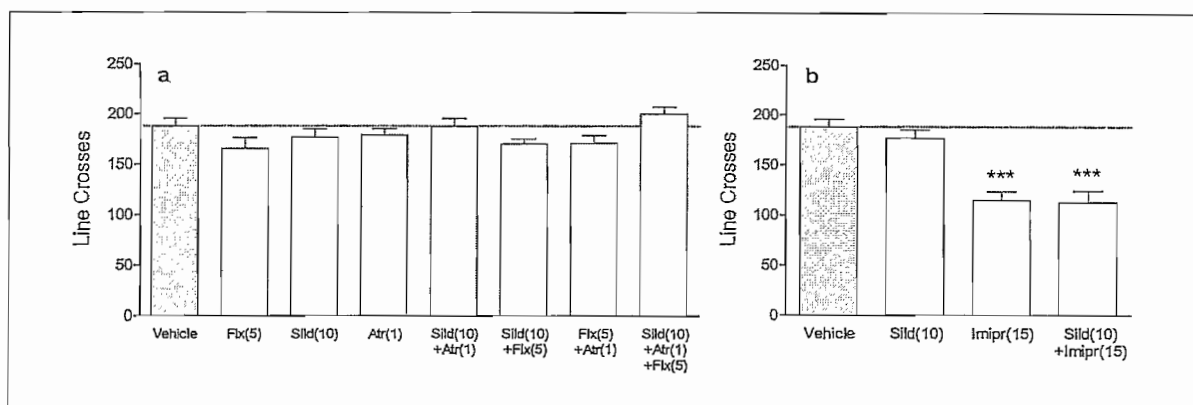


Figure A.6 Locomotor activity measured following a shorter (7 day) treatment period with (a) fluoxetine or (b) imipramine, and combinations of these antidepressants with sildenafil \pm atropine in FSL rats, in terms of the number of line crosses during a 5 minute open field test. Data from (a) and (b) were analysed collectively using a one-way ANOVA, followed by a Tukey-Kramer post-hoc analysis, and are expressed as the mean + SEM (***) $p < 0.001$. All groups consisted of 10-12 rats each ($n = 10-12$), except for the vehicle control group which consisted of 22 rats ($n = 22$). The following abbreviations are used: Flx (fluoxetine); Imipr (imipramine); Sild (sildenafil); Atr (atropine).

A.3 Measurement of mACh receptor density

Aims

Here we explored the involvement of mACh receptors in the antidepressant-like response of sildenafil + atropine. Specifically, we investigated whether sildenafil may up-regulate cortical or hippocampal mACh receptors, which may be involved in the pro-cholinergic action of sildenafil. To this end, radio-ligand saturation binding experiments were performed to measure cortical and hippocampal mACh receptor densities following chronic treatment with sildenafil \pm atropine.

Methods

Drug treatment: A group of FRL rats were injected daily (i.p.) with vehicle (5% DMSO in saline), whereas separate groups of FSL rats were treated daily with vehicle, sildenafil (10 mg/kg), atropine (1 mg/kg) or sildenafil + atropine for 14 consecutive days. Rats were decapitated 12 hours following the final injection. Frontal cortex and hippocampus regions were removed and stored at -80°C until further use.

Isolation of membrane fractions: The brain samples were thawed on ice, weighed and homogenised in 25 ml ice-cold Tris HCl buffer (50 nM, pH = 7). The samples were centrifuged for 10 min at $48,000\times g$ (4°C), the supernatant discarded and the pellet resuspended in 25 ml Tris HCl buffer. The centrifugation step was repeated and the pellet resuspended in an appropriate volume of Tris HCl buffer to yield a dilution of 16 mg wet weight per ml buffer. The protein concentration for each sample was determined using the

Bradford method (Bradford, 1976), and each membrane suspension was diluted with Tris HCl buffer to yield a final protein concentration of 500 µg/ml.

Saturation binding: The suspensions were incubated (in duplicate) with a 7 point concentration series (0.5 to 10 nM) of [³H]-quinuclidinyl (GE Healthcare, Buckinghamshire, England) alone, or in the presence of 1 µM atropine for the measurement of non-specific binding), in a shaking water bath for 15 minutes at 25°C. Following the incubation period, the membrane suspensions were transferred to a Whatman GF/B filter (Merck, Darmstadt, Germany), and washed twice with 5 ml ice-cold Tris HCl buffer to eliminate any unbound ligand. The filters were placed in scintillation vials with 3 ml Filter Count liquid scintillation cocktail (PerkinElmer, Boston, USA) and counted in a scintillation counter.

Statistics: Receptor density was calculated in terms of fmol/mg protein. The data was analysed using one-way ANOVA followed by Tukey-Kramer multiple comparison tests, and a value of $p < 0.05$ was considered statistically significant. The number of rats in each group (n -values) is given in the figure legend.

Results

As illustrated in Figure A.7, there were no significant differences in the mACh receptor density in the cortex [$F(4,16) = 0.74$; $p = 0.58$] or hippocampus [$F(4,13) = 0.22$; $p = 0.92$] of vehicle-treated FRL rats, or FSL rats treated with vehicle, sildenafil, atropine or sildenafil + atropine for 14 days.

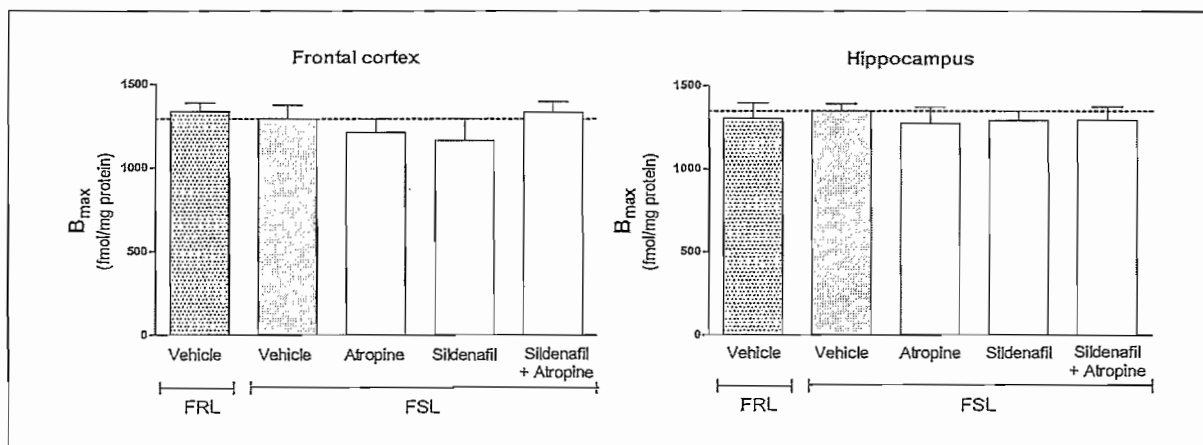


Figure A.7 Muscarinic receptor densities measured in (a) frontal cortex and (b) hippocampus of vehicle-treated FRL rats and FSL rats treated with vehicle, sildenafil, atropine or sildenafil + atropine for 14 days. Data for each graph was analysed separately by using a one-way ANOVA followed by a Tukey-Kramer post-hoc analysis, and are expressed as the mean + SEM. All groups consist of 3-5 experiments each ($n = 3-5$).

Discussion

Although a clear involvement for mACh receptors in the antidepressant-like response of sildenafil is suggested in this study, as well as in a previous report (Brink *et al.*, 2008), the data in Figure A.7 suggests that the sildenafil-cholinergic interaction may not involve alterations in mACh receptor density, but rather some downstream mechanism in the signal transduction pathway of these receptors. An investigation into the specific downstream targets that may be involved is currently underway in our laboratory.

A.4 Synopsis

In summary, although the data presented in this addendum were not considered to have sufficient scientific impact to be submitted to a high-quality peer-reviewed journal, several important findings were made in these experiments. Firstly, we confirmed the genetic dissimilarity between the FSL and FRL colonies in our laboratory in terms of a hypothermic response to a 5-HT_{1A} agonist. Although synergistic interactions were not observed for sildenafil ± atropine and other antidepressants (i.e. fluoxetine and imipramine), the high doses used here may not have allowed such effects to be observed. Of particular interest is the co-administration of sildenafil and imipramine as a possible augmentation strategy in the treatment of depression, given that this drug has antimuscarinic activity and may allow it to act synergistically with sildenafil. In addition, both of these drugs have already been approved for clinical use, making such a clinical evaluation an attractive prospect. Finally, we have shown that the pro-cholinergic action of sildenafil, and the interaction of this drug with atropine in its antidepressant-like response, are not associated with changes in frontal cortical or hippocampal mACh receptor concentration, and implies that the more downstream signalling mechanisms of muscarinic transmission are likely to play a role in these responses.

Addendum B: Instructions to the author

Instructions to the author

Behavioural Pharmacology

Submissions

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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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).

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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

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

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Addendum C: Instructions to the author

Instructions to the author

Behavioural Brain Research

Organization of the article

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[1] Paulus MP, Geyer MP. Three independent factors characterize spontaneous rat motor activity. *Behav Brain Res*, 1993;53: 11-20.

[2] Swanson LW. *Brain Maps: Structure of the Rat Brain*. Amsterdam: Elsevier, 1992, 240 pp.

[3] Van Hoesen GW, Hyman BT. Hippocampal formation: anatomy and the patterns of pathology in Alzheimer's disease. In: Storm-Mathisen J, Zimmer J, Ottersen OP, editors. *Understanding the Brain through the Hippocampus*. Progress in Brain Research, vol 83, Amsterdam: Elsevier, 1990, 445-457.

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Addendum D: Congress contributions

The results obtained in this study were presented at two national congresses (podium presentations), as well as one international congress (poster presentation):

- a) LIEBENBERG N*, BRINK CB, HARVEY BH & BRAND L. 2009. Animal models of depression: Are parallels being drawn responsibly? (Invited paper presented as podium presentation at the 5th International Congress of Pharmacological and Pharmaceutical Sciences (ICPPS), held at North West University, Potchefstroom, North West Province, South Africa, on 28 – 26 September 2009.)
- b) LIEBENBERG N*, BRINK CB, HARVEY BH & BRAND L. 2008. Antidepressant-like properties of sildenafil in a genetic rat model of depression: Role of cholinergic cGMP-interactions. (Paper presented as podium presentation at the 42nd Annual Congress of the South African Society for Basic and Clinical Pharmacology (SASBCP), held at Rhodes University, Grahamstown, Eastern Cape, South Africa, on 2 – 5 October 2008.)
- c) HARVEY BH*, LIEBENBERG N, LIN F, OVERSTREET DH, WEGENER G & BRINK CB. 2008. Antidepressant-like properties of sildenafil in a genetic rat model of depression and effects on marker of cellular resilience. (Paper presented as a poster presentation at the 16th Collegium Internationale Neuro-Psychopharmacologicum Congress, held in Munich, Germany, 13-17 July 2008.)

The abstracts for these presentations are also provided on the following pages.

Animal models of depression: Are parallels being drawn responsibly?

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School of Pharmacy, North West University, Private Bag X6001, Potchefstroom, 2520, South Africa

ABSTRACT

Major depression is an anxiety-related disorder predicted to become the second most debilitating illness after cardiovascular disease by 2020. Current drug treatments of major depression are plagued with bothersome side-effects, a delayed onset of action and a significant percentage of treatment resistance. While the neurobiological basis of depression and the mechanisms of antidepressant action remain elusive, the best described hypotheses include variations of the classical monoaminergic hypothesis of depression. These hypotheses involve dysregulation of adrenergic, serotonergic and dopaminergic neurotransmission in the brain. While cholinergic hypersensitivity, and glutamate and GABA dysfunction have also been postulated to play a role in the neurobiology of depression, newer hypotheses also involve dysregulation of the HPA axis and circadian rhythms, altered neuroplasticity and altered brain reward pathways.

Consequently, there is a need for a better understanding of the neurobiological basis of depression, as well as of the mechanisms underlying the drug treatment of this disorder, especially if we are to discover new targets for drug development. While the human brain and psychological functioning are extremely complex, whether viewed at biological subcellular or systematic level, research into psychiatric disorders remains one of our biggest challenges. Human studies are also complicated by a maze of ethical considerations that often preclude in-depth investigation into the neurobiology of these illnesses. On the other hand, in vitro research becomes too reductionistic to answer complex questions relating to the human mind and psyche, such that the best alternative remains appropriately validated animal simulation models. With these models it becomes possible to investigate behavioural, anatomical, histological, neurobiological and biomolecular changes in depression and drug treatments. However, before this can be attained, the models in use need to demonstrate proven validity for their intended use

Several animal models of depression or models to evaluate antidepressant activity have been developed and validated. These models focus on symptoms of depression (such as anhedonia – i.e. face validity), and/or that they have been demonstrated to be responsive to antidepressant treatment (i.e. predictive validity), and/or that they show some comparison with deviations in anatomical or neurobiological markers associated with depression (construct validity). Examples of these models include the rodent forced swim test, the tail suspension test, behavioural despair, chronic mild stress and olfactory bulbectomized rats. With the evidence of gene X environment interactions in depression, genetic animal models such as the Flinder's sensitive line (FSL) rats are also particularly interesting and useful.

The basis for the validity of these animal models, as well as their value and limitations with respect to depression and antidepressant drug discovery, will be discussed with a focus on the rodent forced swim test. We shall also present recent data on a novel interaction between the NMDA/NO/cGMP pathway and the cholinergic system in producing an antidepressant-like response in this model.

Antidepressant-like Properties of Sildenafil in a Genetic Rat Model of Depression: Role of Cholinergic-cGMP Interactions

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ABSTRACT

Background: The N-methyl-D-aspartate (NMDA)/nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway has been implicated in the neurobiology of depression. Recently we suggested a possible complex interaction between the cholinergic and NO-cGMP pathways in the antidepressant-like response of the phosphodiesterase type 5 (PDE5) inhibitor, sildenafil. Specifically, we demonstrated that chronic (11 days) treatment with a combination of sildenafil (10 mg/kg/day) + atropine (1 mg/kg/day) produces antidepressant-like effects in the forced swim test (FST) in Sprague Dawley rats. Neither of these drugs produced any antidepressant-like effect when administered alone. In the current study, we investigated these findings in a genetic animal model of depression, the Flinders Sensitive Line (FSL) rats. In addition, we evaluated the dose-dependency and onset of action for sildenafil + atropine, as well as the efficacy of various augmentation strategies in combination with fluoxetine and imipramine.

Methods: Treatment regimes: FSL rats were injected intraperitoneally daily with sildenafil (0, 1, 3, 10 and 20 mg/kg/day) ± atropine (1 mg/kg/day), fluoxetine (5 mg/kg/day) or imipramine (15 mg/kg/day) for 14 days. Rats were also treated with sildenafil (10 mg/kg/day) ± atropine in combination with fluoxetine (5 mg/kg/day) or imipramine (15 mg/kg/day) for 7 and 14 days. On the last day of treatment, approximately 5 hours into the dark-cycle (±12 hours following the last injection) immobility was scored during five minutes swim in the FST. In addition, locomotor activity was evaluated in the Open Field Test 2 hours prior to the FST.

Results: Fluoxetine and imipramine separately decreased immobility in FSL rats, comparable to that of FRL control rats, after 14 but not after 7 days. Likewise, when combined with atropine, sildenafil also decreased immobility in FSL rats at doses of 3, 10 and 20 mg/kg/day with a dose-dependent trend, but not with 1 mg/kg/day. While fluoxetine + sildenafil + atropine was not superior to fluoxetine alone, there was a trend for augmentation of imipramine when combined with sildenafil after 14 days. Importantly, only sildenafil + imipramine in combination produced a significant antidepressant-like effect in FSL rats after 7 days. Locomotor activity was unaltered by all treatments, indicating that reduced immobility in the FST was not secondary to increased locomotor activity.

Conclusions: Using a genetic animal model of depression, we have confirmed the antidepressant-like property of sildenafil following “unmasking” by concomitant block of muscarinic receptors. These findings hint at a novel interaction between the cGMP and cholinergic systems in depression, and suggest a strategy for the treatment of depression, using a PDE5 inhibitor in the presence of cholinergic inhibition. Sildenafil-induced augmentation of imipramine, an antidepressant with inherent anticholinergic properties, concurs with this suggestion, and highlights the potential clinical value of this observation..

Antidepressant-like Properties of Sildenafil in a Genetic Rat Model of Depression and Effects on Markers of Cellular Resilience

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ABSTRACT

Depression has been linked to decreased neurogenesis. The NMDA/nitric oxide (NO)/cGMP pathway is implicated in the neurobiology of depression. Recently we suggested possible complex interaction between the cholinergic and NO-cGMP pathways in the anti-depressant-like response of the phosphodiesterase type 5 inhibitor, sildenafil (Brink et al., 2008). Using the forced swim test, we investigated the effects of sildenafil (10 mg/kg/day) ± atropine (1 mg/kg/day) compared to saline and fluoxetine (5 mg/kg/day) for 14 days in Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats. In addition, we examined the effect of sildenafil (1 nM; 10 nM) for 24 hours on specific pro-apoptotic (caspase 3 (Casp3)) and anti-apoptotic markers (Akt, cAMP response element binding protein (Creb), brain-derived neurotrophic factor (BDNF)) in human neuroblastoma cells, using real-time PCR. Fluoxetine, as well as the sildenafil + atropine combination (but not sildenafil or atropine alone) decreased immobility comparable to that of FRL control rats. In neuroblastoma cells, 1 nM, but not 10 nM sildenafil, significantly upregulated genes for Akt, Creb and Casp3, with a tendency for BDNF upregulation. The data confirms antidepressant-like effects of sildenafil + atropine in a genetic animal model of depression. The effects on markers of pro- and antiapoptotic pathways suggest that sildenafil may modulate cellular resilience via modulation of both neuroprotective and neurotoxic pathways. Concluding, chronic sildenafil + atropine treatment, but neither drug alone, displays antidepressant-like properties comparable to that of fluoxetine. Whether modulation of neuroplasticity may be involved in this antidepressant-like response, warrants further investigation.

Abbreviations

Numerical

5-HT _{1A}	Serotonin 1A receptor
5-HT ₂	Serotonin 2 receptor
5-HT ₃	Serotonin 3 receptor
8-OH-DPAT	8-Hydroxy-N,N-dipropyl-2-aminotetralin

A

ACTH	Adrenocorticotropin hormone
ADP	Adenosine diphosphate
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	Analysis of variance
AIDS	Acquired immunodeficiency syndrom

B

BDNF	Brain-derived neurotrophic factor
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C

cGMP	Cyclic guanosine monophosphate
CNG	Cyclic nucleotide-gated ion channels
CREB	cAMP response element binding protein

D

DARRP-32	Dopamine- and cyclic AMP-regulated phosphoprotein
DFP	Diisopropyl fluorophosphate
DG	Dentate gyrus
DMSO	Dimethylsulphoxide
DARRP-32	Dopamine- and cyclic AMP-regulated phosphoprotein

E

ECS	Electroconvulsive shock
ED	Erectile dysfunction
EPM	Elevated plus maze
eNOS	Endothelial nitric oxide

F

FSL	Flinders sensitive line
FST	Forced swim test
FRL	Flinders resistant line

H

HCN	Hyperpolarisation-activated cyclic nucleotide ion channels
HPA	Hypothalamic-pituitary-adrenal
HIV	Human immunodeficiency virus

I

i.p.	Intra-peritoneal
i.c.v.	Intracerebroventricular
IFN- α	Interferon alpha
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-6	Interleukin 6
iNOS	Inducible nitric oxide
IP ₃	Inositol triphosphate

L

LTD	Long-term depression
LTP	Long-term potentiation

M

mACh-R	Muscarinic acetylcholine receptor
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
mGlu	Metabotropic glutamate receptor

N

NAc	Nucleus accumbens
NGF	Neuronal growth factor
NO	Nitric oxide
nNOS	Neuronal nitric oxide
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin 3

P

PFC	Prefrontal cortex
pGC	Particulate guanylyl cyclase
PDE2	Phosphodiesterase type 2
PDE3	Phosphodiesterase type 3
PDE5	Phosphodiesterase type 5
PK-G	cGMP-dependent protein kinase
PTSD	Post-traumatic stress disorder

R

RhoA	Ras homolog gene family member A
RGS2	Regulator of G-protein signalling 2
RGS3	Regulator of G-protein signalling 3
RGS4	Regulator of G-protein signalling 4

S

SEM	Standard error of the mean
SGZ	Hippocampal subgranular zone
SSRI	Selective serotonin reuptake inhibitor
sGC	Soluble guanylyl cyclase
pGC	Particulate guanylyl cyclase

T

TNF- α	Tumor necrosis factor alpha
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V

VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor

W

WHO

World Health Organization

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