Long-lasting bio-behavioural effects of early-life sildenafil administration in stress-sensitive versus healthy control rats

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Abstract

Major depressive disorder (MDD) in children and adolescents is prevalent, serious and of great concern globally. Yet, only two selective serotonin reuptake inhibitors (SSRIs) are approved for the treatment of juvenile MDD, namely fluoxetine and escitalopram. In addition, the effects of early-life exposure to psychotropic drugs on neurodevelopment and the potential long-lasting effects thereof into adulthood are poorly understood. This study investigated the later-in-life bio-behavioural effects of early-life exposure to the psychotropic drug, sildenafil, in stress-sensitive versus healthy control rats.

Male Flinders Sensitive Line (FSL) rats (*n* = 12 per group), a validated genetic animal model of MDD, and behavioural control male Sprague-Dawley (SD) rats (*n* = 12 per group) received either saline or sildenafil (3 mg/kg/day) subcutaneously from postnatal day (PnD) 21 to 34 (for the pre-pubertal groups) or from PnD 35 to 48 (for the pubertal groups) (ethics approval no. **NWU-00277-17-S5**). The rats were subsequently housed under standard laboratory conditions until PnD 60 (i.e. adulthood), representing a wash-out period following sildenafil treatment, leading to only later-in-life, and not immediate, bio-behavioural effects being observed. On PnD 60, a battery of behavioural tests was conducted, consisting of the novel object recognition test (nORT) to assess cognition, the open field test (OFT) to assess general locomotor activity and anxiety-like behaviour, and the forced swim test (FST) to assess depressive-like behaviour. Rats were subsequently euthanized on PnD 60 and hippocampal concentrations of brain-derived neurotrophic factor (BDNF) were measured.

Juvenile sildenafil treatment had no later-in-life effect on cognition, general locomotor activity or anxiety-like behaviour into adulthood in both strains and regardless of treatment initiation age (i.e. pre-pubertal or pubertal). In the FST, saline-treated FSL rats displayed a greater immobility (i.e. enhanced depressive-like behaviour) compared to saline-treated SD rats. Sildenafil treatment reduced the immobility (i.e. reduced depressive-like behaviour) and increased struggling (i.e. enhanced noradrenergic neurotransmission) in the FSL but not in the SD rats (i.e. only in rats genetically susceptible to develop MDD), regardless of treatment initiation age. In addition, sildenafil increased swimming behaviour (i.e. enhanced serotonergic neurotransmission) in the pre-pubertal but not pubertal treated groups (i.e. treatment age susceptibility differences), regardless of the strain. Juvenile sildenafil treatment had no later-in-life effect on hippocampal BDNF concentrations into adulthood in both strains and regardless of treatment initiation age (i.e. pre-pubertal or pubertal).

Our data suggest that both pre-pubertal and pubertal neurodevelopment in rats may be putatively manipulated by sildenafil treatment to bring about long-lasting effects into adulthood.

It can therefore be concluded that early-life sub-chronic sildenafil treatment has later-in-life antidepressant-like effects into adulthood, with no observed later-in-life effect on cognition and anxiety-like behaviour.

Keywords: Major depressive disorder, children, adolescents, sildenafil, neurodevelopment, behavioural tests, phosphodiesterase type 5, Flinders Sensitive Line rat.

Opsomming

Major depressiewe versteuring (MDV) in kinders en adolessente is ’n groot bekommernis wêreldwyd, met slegs twee selektiewe serotonien heropname inhibeerders goedgekeur vir behandeling, naamlik fluoksetien en essitalopram. Daar bestaan verder onduidelikheid oor die effekte wat vroeë lewe behandeling met psigotropiese geneesmiddels op neuro-ontwikkeling het en oor die potensiële blywende effekte daarvan tot in volwassenheid. Die huidige studie het ondersoek ingestel na die blywende biologiese gedragseffekte van vroeë lewe behandeling met die psigotropiese geneesmiddel, sildenafil, in stres-sensitiewe versus gesonde kontrole rotte.

Manlike Flinders Sensitiewe Lyn- (FSL-) rotte (n = 12 per groep), ’n breedvoerig beskryfde en gevalideerde dieremodel van MDV, en manlike Sprague-Dawley- (SD-) rotte (n = 12 per groep) het fisiologiese soutoplossing (salien) of sildenafil (3 mg/kg/dag) ontvang deur daaglikse subkutaneuse inspuitings vanaf postnatale dag (PnD) 21 tot 34 (vir die pre-pubertale groepe) en vanaf PnD 35 tot 48 (vir die pubertale groepe) (etiese goedkeuringsnommer: **NWU-00277-17-S5**). Die rotte was gevolglik onder standaard laboratoriumtoestande gehuisves tot PnD60 (vroeë volwassenheid), wat as ’n uitwasperiode gedien het na die sildenafilbehandeling. Op PnD 60 is die gedragstoetse uitgevoer, naamlik die nuwe voorwerp herkenningstoets (NVHT), oopveldtoets (OVT) en geforseerde swemtoets (GST), om onderskeidelik kognisie (NVHT), lokomotoraktiwiteit en angstigheid (OVT) en depressiewe gedrag (GST) te evalueer. Daarna was genadedood deur dekapitering op PnD 61 toegepas en die konsentrasie brein-verkreë neurotrofiese faktor (BDNF) is in die hippokampus gemeet.

Sildenafilbehandeling het geen effek op kognisie, lokomotoraktiwiteit of angstigheid gehad nie (d.i. in beide FSL- en SD-rotte en ongeag die ouderdom van sildenafilbehandeling). Salien-behandelde FSL-rotte was langer immobiel tydens die GST (d.i. verhoogde depressiewe gedrag) in vergelyking met salien-behandelde SD-rotte. Sildenafilbehandeling het immobiliteit verlaag (d.i. verlaagde depressiewe gedrag) en spartelgedrag verhoog (d.i. verhoogde noradrenergiese neurotransmissie) in die FSL-rotte en nie in die SD-rotte nie (d.i. slegs in die rotte met ’n genetiese vatbaarheid vir die ontwikkeling van MDV), ongeag die ouderdom van sildenafilbehandeling. Verder het sildenafilbehandeling, in beide FSL- en SD-rotte, swemgedrag verhoog (d.i. verhoogde serotonergiese neurotransmissie) in die pre-pubertale en nie in die pubertale behandelingsgroepe nie (d.i. verskille in die ouderdom van behandelingsvatbaarheid). Sildenafilbehandeling het geen effek op BDNF konsentrasies in die hippokampi gehad nie (d.i. in beide FSL- en SD-rotte en ongeag die ouderdom van sildenafilbehandeling). Pre-pubertale en pubertale neuro-ontwikkeling in rotte kan dus moontlik gemanipuleer word om langtermyn effekte tot in volwassenheid tot gevolg te hê. Vroeë lewe

sildenafilbehandeling het dus langtermyn antidepressiewe effekte tot in volwassenheid, met geen effek op kognisie en angstigheid nie.

Sleutelwoorde: Major depressiewe versteuring, kinders, adolessente, sildenafil, neuro-ontwikkeling, gedragstoetse, fosfodiësterase tipe 5, Flinders Sensitiewe Lyn-rot.

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List of abbreviations

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ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase
ACTH	-	Adrenocorticotrophin
AMPA	-	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ANOVA	-	Analysis of variance

B

BBB	-	Blood brain barrier
BDNF	-	Brain-derived neurotrophic factor

C

cAMP	-	Cyclic adenosine monophosphate
cGMP	-	Cyclic guanosine monophosphate
CI	-	Confidence interval
CNS	-	Central nervous system
COMT	-	Catechol-O-methyltransferase
CREB	-	Cyclic adenosine monophosphate response element binding protein
CRH	-	Corticotrophin-releasing hormone

D

DFP	-	Diisopropyl fluorophosphate
DNA	-	Deoxyribonucleic acid

E

ECT	-	Electroconvulsive therapy
EDTA	-	Ethylenediaminetetraacetic acid

EGTA	-	Ethylene glycol tetraacetic acid
ELISA	-	Enzyme-linked immunosorbent assay
EPM	-	Elevated Plus Maze

F

FC	-	Frontal cortex
FDA	-	Food and Drug Administration
FGF	-	Fibroblast growth factor
FRL	-	Flinders Resistant Line
FSL	-	Flinders Sensitive Line
FST	-	Forced Swim Test

G

GABA	-	Gamma-Aminobutyric acid
GTP	-	Guanosine-5'-triphosphate

H

HPA	-	Hypothalamic-pituitary-adrenal
HPA axis	-	Hypothalamic-pituitary-adrenal axis
HPLC	-	High performance liquid chromatography
HPLC-EC	-	High performance liquid chromatography with electrochemical detection

I

IDO	-	Indoleamine 2, 3-dioxygenase
IGF	-	Insulin-like growth factor
IL	-	Interleukin
IL-1	-	Interleukin 1
IL-6	-	Interleukin 6

K

KA	-	Kynurenic acid

L

LC-NE	-	Locus-coeruleus–norepinephrine
LTD	-	Long-term depression
LTP	-	Long-term potentiation

M

mAChR	-	Muscarinic acetylcholine receptor
MAO	-	Monoamine oxidase
MAOI	-	Monoamine oxidase inhibitor
MDA	-	Malondialdehyde
MDD	-	Major Depressive Disorder
MDE	-	Major Depressive Episode
mGluR	-	Metabolic glutamate receptor
MHRA	-	Medicines and Healthcare products Regulatory Authority
mRNA	-	Messenger ribonucleic acid

N

nAChR	-	Nicotinic acetylcholine receptor
NARI	-	Noradrenaline reuptake inhibitor
ND	-	Natal day
NERT	-	Noradrenalin reuptake transporter
NMDA	-	N-methyl-D-aspartate
nNOS	-	Neuronal nitric oxide synthase
NO	-	Nitric oxide
nORT	-	Novel Object Recognition Test
NP	-	Natriuretic peptides
NSAID	-	Non-steroidal anti-inflammatory drug

O

OCD	-	Obsessive compulsive disorder
OFT	-	Open field test

P

PDE5	-	Phosphodiesterase type 5
PFC	-	Prefrontal cortex
PK-G	-	Protein kinase G
PnD	-	Postnatal day
PI	-	Preference index
PVN	-	Paraventricular nucleus

Q

QA	-	Quinolinic acid

R

REM	-	Rapid eye movement
ROS	-	Reactive oxygen species
RNS	-	Reactive nitrogen species

S

sc	-	Subcutaneous
SD	-	Sprague-Dawley
SEM	-	Standard error of the mean
sGC	-	Soluble guanylyl cyclase
SNRI	-	Serotonin-noradrenaline reuptake inhibitor
SOD	-	Superoxide dismutase
SSRI	-	Selective serotonin reuptake inhibitor

T

TCA	-	Tricyclic antidepressant
TDO	-	Tryptophan 2, 3-dioxygenase
TNF-α	-	Tumour necrosis factor alpha

V

VEGF	-	Vascular endothelial growth factor

W

WHO	-	World Health Organisation
WMH	-	World Mental Health

Declaration by student

I, Juandré Lambertus Bernardus Saayman, hereby declare that all the literature research, experimental work and data capturing and interpretation of this study were conducted by myself. I further declare that the initial version of this dissertation was also written by myself, and that improvements and corrections were then made as per advice from study guidance. My supervisor (Prof. Christiaan B Brink) funded this project with grants obtained from the National Research Foundation (NRF - grant no. 103371 IFR160118156926) and Medical Research Council (MRC) and both he and the co-supervisor (Dr. Stephanus F Steyn) assisted me with the interpretation of the data obtained from the experimental work that was conducted and proof read this dissertation in preparation for the final version. All neurochemical analyses were conducted by myself, with assistance from a senior laboratory technician (Mr. Walter Dreyer) and my assistant supervisor (Mr. Francois P Viljoen). All the statistical analyses were conducted by myself, with guidance from my co-supervisor (Dr. Stephanus F Steyn) and Mrs. Marike Cockeran from the Statistical Consultation Services of the North-West University.

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Chapter 1. Introduction

The following introductory chapter serves as a guide to the dissertation (i.e. broad outline) and to the study as a whole and provides a sense of direction to the reader. Therefore, this chapter is very condensed, followed by a more thorough and elaborate discussion of the relevant literature in *Chapter 2*.

1.1 Dissertation layout

This dissertation is written and submitted in the standard *"article"*-format for dissertation submission, as approved by the North-West University. The format outline serves to assist the reader in finding key elements of the study inside the dissertation and is as follows:

Chapter 1: **Introduction**.

Chapter 2: **Literature review** of scientific study findings and reviews relevant to the current study to create a general background and understanding from which the results of this project can be interpreted.

Chapter 3: **Manuscript** (article-format) of the study, for submission to an accredited international journal. Important to note is that the manuscript contains the main findings of the project and is prepared in-line with the guidelines of the journal, and may therefore contain different referencing, compared to the rest of the dissertation

Chapter 4: **Summary**, c**oncluding remarks** and **suggestions** for further investigations.

Addendum A: Additional **materials and methods**, not included in the manuscript.

Addendum B: Additional **data**, not included in the manuscript.

Addendum C: Abstract of a **podium presentation** of the data from this study at a national congress, as well as proof of attendance.

Addendum D: **Ethics approval** letter

The reference list of the manuscript is presented at the end of the manuscript (i.e. Chapter 3) and is in accordance with the specific reference style required by the scientific journal to which the manuscript will be submitted. All of the other referencing throughout this dissertation was done with EndNote X8 software, is cited according to the Harvard style (preferred by the North-West University) and can be found at the end of *Chapter 4*.

This dissertation is written in United Kingdom (UK) English.

1.2 Problem statement

Major depressive disorder (MDD) is a globally prevalent (O'Donnell & Shelton, 2011), debilitating and serious neuropsychiatric disorder (NIMH, 2011). MDD has a low remission rate and precipitates reduced quality of life, increased suicide risk, impaired cognitive and social functioning, decreased work performance and a considerable economic burden on the affected individual's family, employer and society at large (Sobocki *et al.*, 2006; Lépine & Briley, 2011; American Psychiatric Association, 2013; Zhang *et al.*, 2016; Johnston *et al.*, 2018). Individuals suffering from MDD also experience a number of physical and psychological symptoms that may prove to be a lifelong challenge for these individuals (see section 2.3 for symptoms) (O'Donnell & Shelton, 2011; Kemp *et al.*, 2012). Children with an MDD-diagnosed parent (especially maternal MDD) are associated with having a greater risk for impaired development (e.g. difficulties with affect regulation, behavioural and emotional difficulties and maladaptive social interactions) and the development of psychiatric disorders. Such adverse effects may potentially have long-lasting consequences for the psychiatric health of the child, and even future generations (Lépine & Briley, 2011). Alarmingly, over 320 million people globally suffer from MDD (World Health Organization, 2017a), which may be an underestimation due to misdiagnosis and/or underreporting. Moreover, in our own country (South Africa), the lifetime prevalence of MDD is also of concern and is estimated to be as high as 10.0% (Tomlinson *et al.*, 2009; Kessler & Bromet, 2013). The lifetime prevalence of MDD in South Africa is comparable to that of other developing countries (e.g. 8.0% for Mexico, 9.0% for India and 10.9% for Lebanon), whereas developed countries appear to have a higher lifetime prevalence of MDD (e.g. 17.9% for the Netherlands, 19.2% for the United States of America and 21.0% for France) (Bromet *et al*., 2011).

MDD also has an alarming impact on juveniles (i.e. children and adolescents), affecting 2.5% of pre-adolescent children, therefore being the most common psychiatric disorder in this age group (Bylund & Reed, 2007). Moreover, paediatric MDD poses a fourfold increased risk of recurring

during adulthood (Pine *et al.*, 1998), is an important predictor of subsequent childhood psychiatric disorders (including anxiety disorders and long-term MDD) later in life and is also related to long-lasting psychosocial impairment and poor work performance into adulthood (Bufferd *et al.*, 2012). Furthermore, severe MDD frequently leads to suicide, not only in adults, but also in juveniles (World Health Organization, 2017a), with suicide being the fourth leading cause of death in pre-adolescent children globally (Hulvershorn *et al.*, 2011b).

Most, if not all, antidepressants currently available present with safety and efficacy concerns, as well as a slow onset of antidepressant action (O'Donnell & Shelton, 2011; Sadaghiani *et al.*, 2011). Furthermore, only fluoxetine and escitalopram, both selective serotonin reuptake inhibitors (SSRIs), have been shown to be effective in the treatment of paediatric MDD and have been approved for this indication. In addition, the United States of America Food and Drug Administration (FDA) has issued a "black-box" warning of an initial increased risk of suicidal ideation in juveniles treated with SSRIs (Klomp *et al.*, 2014). Therefore, novel pharmacological treatment strategies are needed to treat paediatric MDD, especially considering that the prescription rates for selective serotonin reuptake inhibitors (SSRIs) have increased dramatically in this age group (Zito & Safer, 2001; Zito *et al.*, 2002; Steinhausen & Bisgaard, 2014; Steinhausen, 2015).

Fluoxetine has been approved for the treatment of MDD in children 8 years and older, whereas escitalopram has been approved for the treatment of MDD in adolescents 12 years and older (Soutullo & Figueroa-Quintana, 2013). Similar to that seen in adults, remission rates are extremely low in juveniles (Marais *et al.*, 2009). Nevertheless, antidepressants remain the first line treatment in moderate and severe MDD (Willner *et al.*, 2013), despite the above-mentioned concerns, whereas in mild MDD non-pharmacological interventions (psychotherapy, life-style changes and support groups) are used as first line therapy, either as an augmentation strategy or monotherapy. Even though there is an immediate increase in the serotonin concentrations within the synaptic cleft following SSRI treatment, the therapeutic effect can only be seen after 3-4 weeks and remission only after 6-8 weeks of treatment (a more detailed discussion follows in section 2.7.1.), further highlighting the need for novel treatment strategies, with a more rapid onset of antidepressant action, to treat paediatric MDD.

MDD has furthermore been associated with both an environmental and a genetic origin (Nestler *et al.*, 2002; Kiyohara & Yoshimasu, 2009b). In addition to the well-described role of both genetics (Rice *et al.*, 2002) and environmental impact (Eley & Stevenson, 2000) on depressive symptoms, behavioural genetic research has provided confirmation of interactions between individual (genetic, biological or familial) vulnerability and environmental stress (Silberg *et al.*, 2001; Eley *et al.*, 2004). In this regard, interactions between environmental factors and a

genetic susceptibility to develop MDD are suggested to result in MDD and this is known as the gene-environment hypothesis of MDD (see section 2.5.1.1) (Lesch, 2004). This is further illustrated by the observation that two different genotypes respond to environmental variation in different ways (Davies *et al.*, 2012).

A number of brain regions are associated with MDD (NIMH, 2011) and in severe cases morphological alterations in these regions manifest as an enlargement of the amygdala, reduction in the size of the hippocampus, neurodegeneration and/or impaired neuroplasticity (Pittenger & Duman, 2008; Kemp *et al.*, 2012). The aforementioned have been associated with impaired hippocampal and prefrontocortical activity and neurocognitive abnormalities, viz. impaired memory, indecisiveness and poor concentration (Pittenger & Duman, 2008; Kemp *et al.*, 2012), that may persist even after symptoms of MDD have subsided (Solé *et al.*, 2015).

Several hypotheses for the neurobiological basis of MDD exist which collectively point to a number of physiological and neurological systems, viz. *monoaminergic* (Schildkraut, 1965; Katzung, 2007b), *cholinergic* (Janowsky *et al.*, 1972) and *glutamatergic* (Sanacora et al., 2012) pathways in the brain, the hypothalamic-pituitary-adrenal axis (HPA axis) (Sheline *et al.*, 1996; Mizoguchi *et al.*, 2003), immunological systems (e.g. inflammation), as well as *neuroplasticity*, to name a few. A more elaborate discussion of the relevant hypotheses for the neurobiological basis of MDD follows in *Chapter 2*.

However, the current study focusses on the *nitric oxide-cyclic guanosine monophosphate* (NO-cGMP) pathway and how areas within this neurological pathway may present as novel targets for the treatment of MDD, in particular paediatric MDD. The NO-cGMP pathway has been investigated for its role in the development of MDD in several studies (Harvey, 1996; Wang & Robinson, 1997; Harvey, 2006; Dhir & Kulkarni, 2007; Brink *et al.*, 2008; Feil & Kleppisch, 2008; Puzzo *et al.*, 2008), which have suggested that when this pathway is stimulated or inhibited, neurochemical alterations are seen in the brain, affecting crucial neurological constructs, as well as monoaminergic regulation and function (Feil & Kleppisch, 2008). Results have suggested different (sometimes conflicting) roles for the NO-cGMP pathway. Nevertheless, these neurochemical alterations, along with an increase in neuroplasticity (Feil & Kleppisch, 2008; Puzzo *et al.*, 2008), form the basis for the proposed antidepressant effect specifically seen with phosphodiesterase type 5 (PDE5) inhibitors.

PDE5 inhibitors are compounds of relevance, due to their modulating effect on the NO-cGMP pathway (Liebenberg *et al.*, 2010a). Importantly, the antidepressant-like effects of sildenafil (i.e. a PDE5 inhibitor) were first demonstrated in preclinical studies in our laboratories (Brink *et al.*, 2008; Liebenberg *et al.*, 2010a; Liebenberg *et al.*, 2010b). These antidepressive-like effects

were later confirmed by various other independent laboratories (Baek *et al.*, 2011b; Matsushita *et al.*, 2012; Tomaz *et al.*, 2014; Wang *et al.*, 2014c; Socała *et al.*, 2016). Interestingly, high doses of sildenafil (≥ 10 mg/kg/day) require co-administration of a centrally acting antimuscarinic drug (atropine) to induce antidepressant-like effects (Brink *et al.*, 2008; Liebenberg *et al.*, 2010a). It is proposed that sildenafil's lack of antidepressive-like effects at higher concentrations is due to its cholinotropic actions (depressogenic effect) in addition to its ability to elevate cyclic guanosine monophosphate (cGMP) concentrations (antidepressive-like effect) in the central nervous system (Brink *et al.*, 2008). Therefore, the antidepressive-like effects of sildenafil (due to an elevation in central cGMP concentrations) are "masked" by the simultaneous elevation in cholinergic neurotransmission (see section 2.5.1.2) (Brink *et al.*, 2008).

Moreover, anxiolytic-like effects for sildenafil and tadalafil, both PDE5 inhibitors, have been demonstrated in a previous study conducted in rodents in our laboratories (Liebenberg *et al.*, 2012). In addition to the antidepressive- and anxiolytic-like effects of PDE5 inhibitors, pro-cognitive effects have also been shown by studies conducted on sildenafil and tadalafil in both rodents (Rutten *et al.*, 2007; Rutten *et al.*, 2009; Baek *et al.*, 2011b; García-Barroso *et al.*, 2013) and non-human primates (Rutten *et al.*, 2008a). Also, sildenafil has been shown to augment the antidepressant-like effects of atypical antidepressants in rodents (Socała *et al.*, 2012).

Sildenafil has been used extensively in neonates, infants, and children for the off-label treatment of pulmonary arterial hypertension associated with diverse heart and lung diseases (Humpl *et al.*, 2005; Mourani *et al.*, 2009). Therefore, the potential use of sildenafil in juveniles suffering from MDD is feasible.

Furthermore, there is great concern about the potential long-lasting effects of early-life treatment with a psychotropic drug and the possible effects on neurodevelopment. Neurodevelopment is a complex process and pharmacological treatment during this period may permanently alter the brain's functional integrity in adulthood (Gomes da Silva *et al.*, 2012). A previous study has shown a significant impact of early-life antidepressant treatment on neurodevelopment, influencing neurobiological functioning in adulthood and often resulting in not only enhanced depressive-like behaviour, but also enhanced anxiety-like behaviour (De Jong *et al.*, 2006), whereas a previous study in our laboratories has shown the contrary with regards to depressive-like behaviour (Steyn, 2011).

Therefore, PDE5 inhibitors have the potential to be effective in the treatment of paediatric MDD, with possible long-lasting, favourable effects into adulthood. If PDE5 inhibitors, as a novel treatment modality, prove effective, they may have a great impact on the future of

neuropsychopharmacology, not only as a feasible and novel treatment option for MDD, but also for other neuropsychiatric illnesses. The current study therefore investigated the later-in-life effects of sub-chronic pre-pubertal and pubertal (time of ongoing neurodevelopment) administration of the psychotropic drug, sildenafil, on behaviour and on brain levels of a biomarker of neuroplasticity and depression, as it manifests during adulthood.

1.3 Study objectives

1.3.1 Primary objective

To investigate in a translational genetic animal model of depression (the FSL rat) whether pre-pubertal (PnD 21-34) and/or pubertal (PnD 35-48) sub-chronic administration of sildenafil (PDE5 inhibitor), versus vehicle-control, exerts any later-in-life bio-behavioural effects, as displayed (after wash-out) in adulthood (PnD 60), including modulation of natural depressive-like behaviour, cognition and neurobiological markers of depression.

1.3.2 Secondary objectives

- To investigate the role of genetic susceptibility in any later-in-life neurobehavioural effects of sildenafil, by comparing its effects in stress-sensitive FSL rats to those observed in normal SD rats; and
- To investigate the role of juvenile age of sildenafil administration on any later-in-life neurobehavioural effects, by comparing sub-chronic administration of sildenafil during pre-puberty to the effects observed following sub-chronic administration during puberty.

1.4 Study layout

In the current study sildenafil or vehicle-control (i.e. saline) was administered to male FSL and male SD rats between PnD 21-34 for the pre-pubertal groups and between PnD 35-48 for the pubertal groups. An illustration of the study layout can be seen in **Figure 1-1** below.

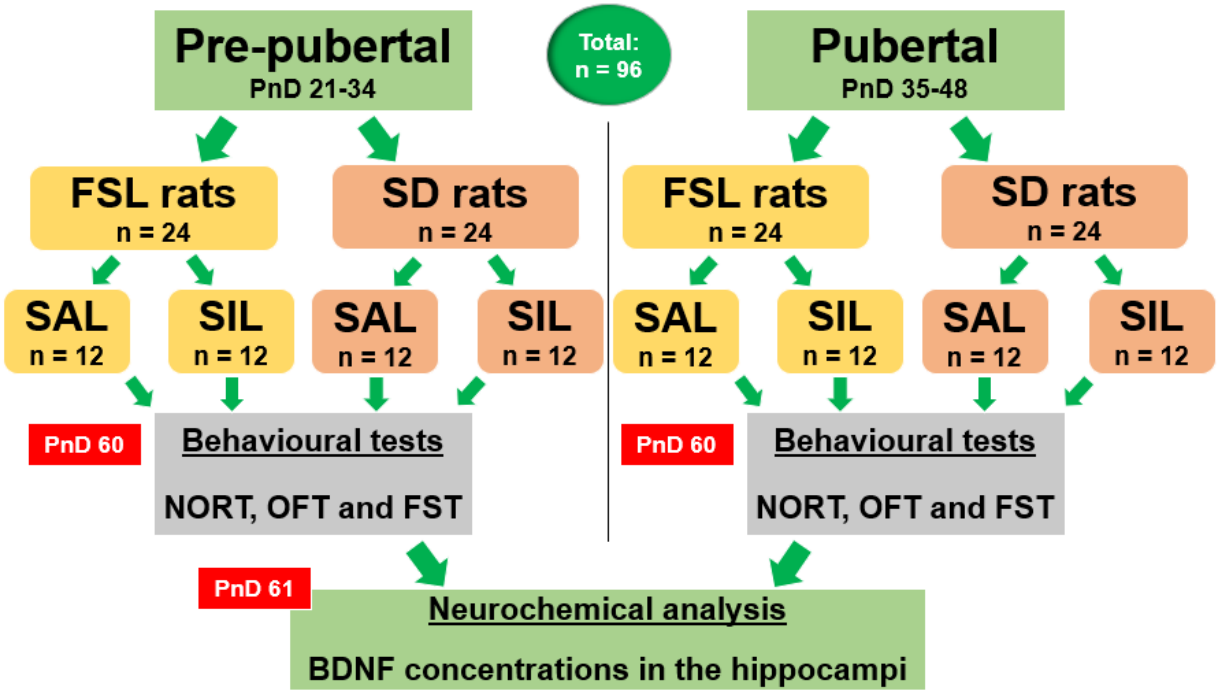

Figure 1-1: A schematic illustration of the study layout. With abbreviations: PnD = postnatal day, n = number of rats, FSL = Flinders Sensitive Line rats, SD = Sprague-Dawley rats, SIL = sildenafil, SAL = saline, NORT = novel object recognition test, OFT = open field test, FST = forced swim test, and BDNF = brain-derived neurotrophic factor.

Treatment groups consisted of 12 rats each and the rats received either sildenafil or vehicle control via daily subcutaneous (sc) injection for 14 days:

Pre-pubertal groups	Pubertal groups
12 FSL rats → Vehicle-control	12 FSL rats → Vehicle-control
12 FSL rats → Sildenafil	12 FSL rats → Sildenafil
12 SD rats → Vehicle-control	12 SD rats → Vehicle-control
12 SD rats → Sildenafil	12 SD rats → Sildenafil

Thereafter, all the rats were housed under standard laboratory conditions until PnD 60, when locomotor activity, anxiety-like behaviour, depressive-like behaviour and cognition were evaluated by a battery of behavioural tests, as outlined in **Table 1-1** below.

Table 1-1: The battery of behavioural tests that were conducted on PnD 60, with the parameter(s) measured by each test.

Behavioural test	Parameter(s) measured
Novel object recognition test (NORT)	• Cognition (memory)
Open field test (OFT)	• Locomotor activity • Anxiety-like behaviour
Forced swim test (FST)	• Depressive-like behaviour • Serotonergic and noradrenergic neurotransmission

The above-mentioned battery of behavioural tests was conducted in the order as presented in the table, to ensure that the least stressful tests are executed first and that the most stressful test is executed last. We have previously demonstrated that when performing the battery of behavioural tests in this order, subsequent tests are not affected by the former tests (Mokoena *et al.*, 2015). Within 24 hours after the behavioural tests were completed, the rats were euthanized, and their hippocampi collected for subsequent BDNF analysis.

1.5 Hypothesis

Based on current literature, we hypothesise the following:

- Sub-chronic administration of the PDE5 inhibitor, sildenafil, to FSL rats (a genetic animal model of depression) during pre-puberty and puberty will have later-in-life effects into adulthood, compared to vehicle control-treated animals, to:
  - reduce depressive-like behaviour;
  - reduce anxiety-like behaviour; and
  - enhance impaired cognition;
- Sub-chronic administration of the PDE5 inhibitor, sildenafil, to FSL rats during pre-puberty and puberty will increase the brain-derived neurotrophic factor (BDNF) concentration within the hippocampus, as observed in adulthood;
- The above-mentioned later-in-life effects of sildenafil will not be seen in SD rats, thereby demonstrating the role of genetic susceptibility; and
- The later-in-life effect of juvenile sildenafil administration will be comparable between groups treated during the pre-pubertal versus pubertal phases, but effects on serotonergic-mediated behaviour (i.e. swimming behaviour) will be more pronounced in

the pre-pubertal group compared to the pubertal group where this system is already mature.

1.6 Expected impact

We expect the following outputs:

- Presentations & publications
  - One podium presentation at a national congress
  - One article in an accredited international journal
  - One M.Sc. dissertation
- Training
  - One M.Sc. student
- Study outcomes
  - Contribute to current knowledge regarding
    - ✓ our understanding of the later-in-life psychotropic and neurodevelopmental effects of juvenile treatment with PDE5 inhibitors
    - ✓ the role of genetic susceptibility in any later-in-life effects of PDE5 inhibitors
    - ✓ providing cues for further investigating the potential clinical use of PDE5 inhibitors (sildenafil) in the treatment of paediatric (juvenile) MDD

1.7 Ethical considerations

All the animal procedures in this study were approved by the NWU-AnimCare Animal Research Ethics Committee (NHREC reg. number AREC-130913-015), Faculty of Health Sciences, North-West University (approval number: **NWU-00277-17-S5**). Animals were bred, supplied and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019; AAALAC accreditation international file #1717) of the Pre-Clinical Drug Development Platform (PCDDP) of the North-West University. All animals were maintained, and all procedures performed in studies involving animals were in accordance with the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation. Moreover, the researcher that handled the animals received appropriate training and completed an animal handling course. Also, animals were handled under the supervision of a veterinarian and laboratory animal technicians.

Furthermore, all the experiments and procedures involving animals in this study were conducted according to a research proposal (containing valid and accepted methods) that was approved by the relevant research committee (Translational Neuroscience, Faculty of Health Sciences,

North-West University). In addition, experiments and procedures involving animals also adhered to the guidelines outlined in the South African National Standards: The care and use of animals for scientific purposes (SANS 10386:2008) and the experimental data are furthermore reported according to the National Centre for the Replacement, Refinement and Reduction of Animals in Research's Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines (Kilkenny *et al.*, 2010). In this regard, the minimum number of animals (more than a minimum of 5 animals, as outlined in the ARRIVE guidelines (Kilkenny *et al*., 2010)) needed for statistically significant results were used, as estimated by an evidence-based estimation (when sufficient experience exists and as published, with similar animal species, type of measurements and study design) (Liebenberg *et al*., 2010). In this regard, assistance was provided by a statistician (Mrs. Marike Cockeran) from the Statistical Consultation Services of the North-West University.

This study adhered to the 3R principle for preclinical research:

Replace: Behaviour, the developing brain and associated neurobiochemistry are implicated in MDD and form part of particularly complex systems. As a result, *in vivo* animal models cannot be replaced with simple, non-sensory models (e.g. computerised models and lower order invertebrates). In addition, careful consideration was also given to the selection of the strains of rats (FSL and SD) used in this study, based on a comprehensive literature review. Although MDD has a significant prevalence in women, the use of female rats in a translational animal model of MDD poses a well-known complexity when considering biological and physiological variances caused by the oestrous cycle (Slattery & Cryan, 2014) and include discrepancies in drug metabolism (Kokras *et al.*, 2011), oxytocin receptor expression (Bale *et al.*, 1995) and HPA axis activity (Atkinson & Waddell, 1997). This may influence the physiological and psychological stress response (Marusak *et al.*, 2015), resulting in the use of male animals only by the majority of preclinical studies (Slattery & Cryan, 2014). Therefore, only male rats were used in this study.

Refine: All the experiments and procedures involving animals in this study were conducted according to validated and accepted methods. The layout of this study was structured in such a way as to prevent the duplication of data and animal numbers were empirically based.

Reduce: Only the number of animals required for statistically significant results were used in this study.

Moreover, the use of animal models to investigate possible novel treatment strategies for MDD is justified by the serious nature of MDD and the great suffering experienced by individuals affected by this disorder, including children and adolescents (positive cost to benefit ratio). The general welfare of the animals was monitored daily by making use of monitoring sheets and

humane endpoints were established before commencing with this study, ensuring that the animals did not experience more stress and/or distress than expected and approved by the Animal Research Ethics Committee. Animal welfare was the primary consideration during studies that were conducted on animals.

Chapter 2. Literature review

This chapter provides an extensive literature review on matters relevant to major depressive disorder (MDD), with a focus on paediatric MDD, and will cover aspects such as the epidemiology, signs and symptoms, current diagnostic criteria, and aetiology of MDD, a more elaborate discussion on the involvement of the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) system in MDD, novel antidepressant targets and existing therapeutic options and animal models of depression.

2.1 Major depressive disorder

Depressed mood is experienced by nearly all people at some point during their lives and may be a perfectly normal response to stressful events (Bylund & Reed, 2007). However, when excessive, inappropriate stress responses impair normal function, it becomes a dire clinical disorder (Bylund & Reed, 2007). Indeed, when symptoms of depressed mood persist and become debilitating, disproportionate to the stressor or even with no direct cause, the condition is known as MDD (Trivedi *et al.*, 2006b). MDD affects people of all ages, race and economic classes, impacts nearly all aspects of a person's existence, including the individual's psychological, social, mental and biological wellbeing, and affects not only the individual but also people around him/her, as well as their work environment and productivity (Trivedi *et al.*, 2006b).

To further complicate this global problem, only one-third of all MDD patients treated with a single antidepressant achieve total remission and another third of all patients remain unresponsive to a second or further antidepressant treatment attempts, a condition known as treatment-resistant MDD (Trivedi *et al.*, 2006b). In addition to the efficacy concerns and a delayed onset of antidepressant action (Hindmarch, 2001; McIntyre & O'Donovan, 2004; Machado-Vieira *et al.*, 2017), antidepressants also have troublesome side-effect profiles, including weight gain, agitation, dizziness, headache, dry mouth, nausea, diarrhoea, sexual dysfunction and sleep disturbances (Hindmarch, 2001; Richelson, 2001; Clayton *et al.*, 2002; Masand & Gupta, 2002; Ashton *et al.*, 2005; Lam *et al.*, 2012). These unfavourable side effects

contribute to a high incidence of non-adherence to antidepressant therapy (Serna *et al.*, 2010; Hung *et al.*, 2011).

Furthermore, the use of antidepressants is associated with an initial increase in suicidal ideation and behaviour, especially in children and adolescents (Jick *et al.*, 2004). In fact, as previously mentioned, the United States of America Food and Drug Administration (FDA) has issued a "black-box" warning of an initial elevated risk of suicidal thoughts and behaviour in children and adolescents treated with antidepressants, in particular treatment with selective serotonin reuptake inhibitors (SSRIs) (Jick *et al.*, 2004; Wessely & Kerwin, 2004; Klomp *et al.*, 2014).

To summarise, MDD poses a significant and serious global challenge, with inadequate treatment modalities, warranting extensive research to gain a better understanding of the condition and to acquire better treatment options or other solutions.

2.1.1 Major depressive disorder in children and adolescents

Childhood depression has become a major concern globally and it has been reported to be the most common psychiatric disorder in children (Bylund & Reed, 2007). The possibility that children may be affected by MDD was once believed to be improbable, solely based on the assumption that children cannot be prone to extremes in mood (Basu & Reddi, 2012). Only by the late 1990s did epidemiological studies demonstrate that MDD can in fact affect children (Weissman *et al.*, 1999). This led to an increase in the diagnosis of childhood MDD, as well as higher associated antidepressant (i.e. SSRIs) prescription rates (Zito *et al.*, 2002), altogether resulting in an increased susceptibility to both beneficial and harmful effects of antidepressant use (Andersen & Navalta, 2004; Branchi, 2011), including both immediate and long-lasting neurodevelopmental effects. Little is known about the possible long-lasting neurodevelopmental effects of antidepressants (and other central acting drugs) during the vulnerable stages of the developing brain. For this reason, more research into these possible long-lasting effects of early-life psychotropic drugs are warranted.

Furthermore, about 25% of children will suffer from at least one major depressive episode (MDE) before they reach adulthood (Kessler *et al.*, 2001). MDD in children and adolescents has been related to memory impairments (Günther *et al.*, 2004), very low self-esteem (Stavrakaki *et al.*, 1991; Renouf *et al.*, 1997) and an elevated risk of suicidal behaviours (Weissman *et al.*, 1999; Fava & Kendler, 2000; World Health Organisation, 2012), making suicide the leading cause of death in juveniles worldwide (Hulvershorn *et al.*, 2011b; World Health Organization, 2017b), and the abuse of substances (Lubman *et al.*, 2007). These consequently influence academic and social development and functioning (Wagner, 2005).

2.2 Epidemiology

It was estimated that the proportion of the global population that suffered from MDD in the year 2015 was 4.4% (World Health Organization, 2017a). The prevalence of MDD varies by World Health Organisation (WHO) Region, from a low of 2.6% in males in the Western Pacific Region to a high of 5.9% in females in the African Region (World Health Organization, 2017a) and the variation according to WHO Region is illustrated in **Figure 2-1** below.

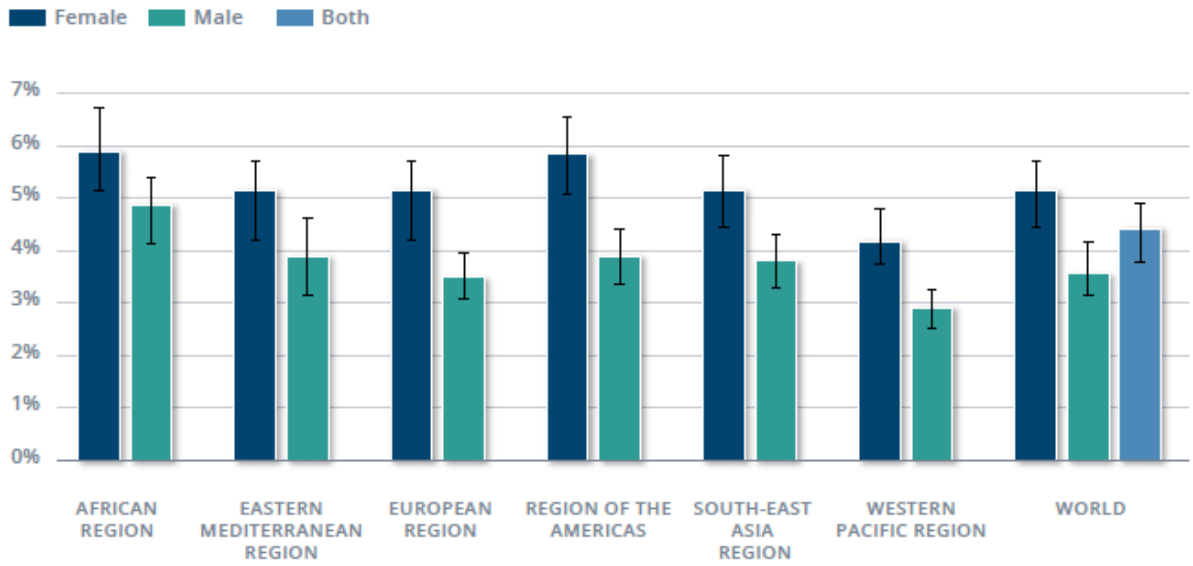

Figure 2-1: Prevalence of MDD (% of regional population), by WHO Region (World Health Organization, 2017a).

Prevalence rates vary by age, reaching a peak in older adulthood (above 7.5% amid females between the ages of 55 to 74 years, and above 5.5% amid males in the same age group) (World Health Organization, 2017a). MDD also occurs in children and adolescents younger than 15 years of age, but at a lower rate than in older age groups. Furthermore, MDD is more prevalent in women (5.1%) than in men (3.6%) (World Health Organization, 2017a), and the global prevalence of MDD, by age and sex, can be seen illustrated in **Figure 2-2** below.

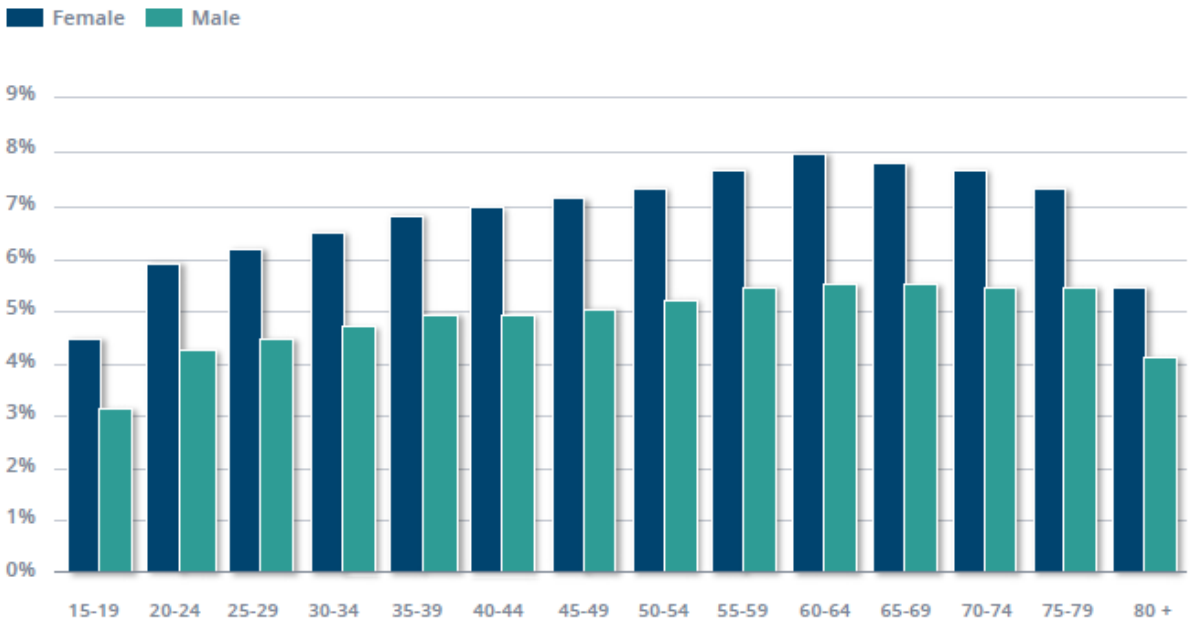

Figure 2-2: The global prevalence of MDD, by age and sex (%) (World Health Organization, 2017a).

The total number of people suffering from MDD is estimated to be 322 million globally (World Health Organization, 2017a) and the total approximated number of people suffering from MDD increased by 18.4% between 2005 and 2015 (Vos *et al.*, 2016), this is a reflection of the overall growth of the global population and a proportionate elevation in the age groups at which MDD is more prevalent. **Figure 2-2** further indicates the high prevalence of MDD in adolescents, with a greater prevalence of MDD in young women compared to young men (World Health Organization, 2017a). The total number of cases of MDD by WHO Region is illustrated in **Figure 2-3** below.

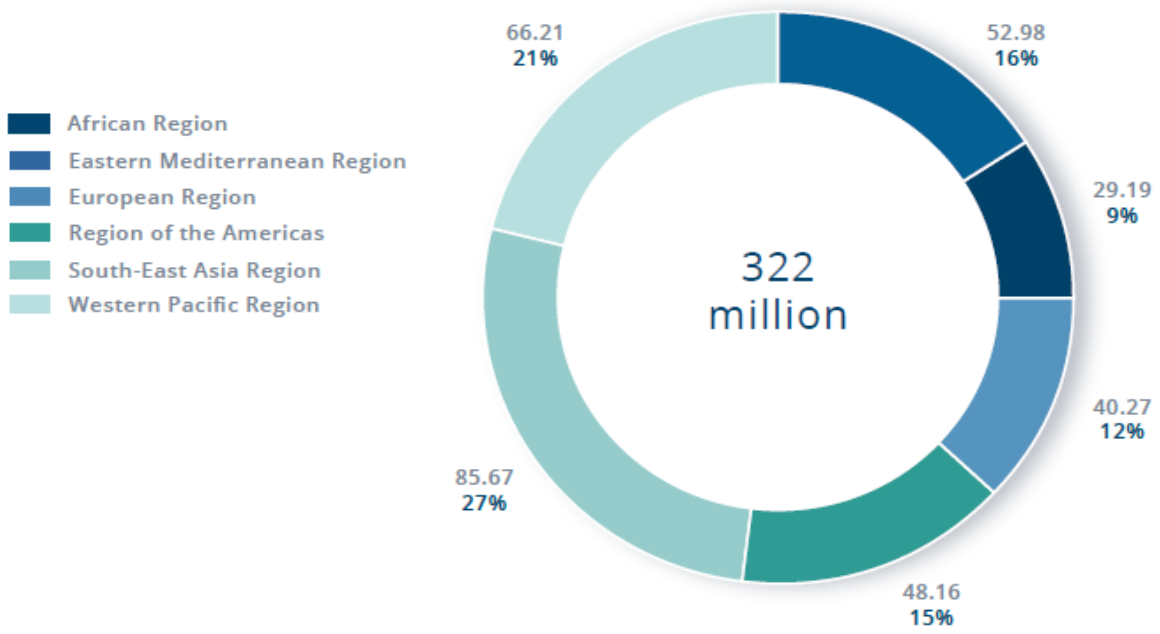

Figure 2-3: Cases of MDD in millions (% of global population), by WHO Region (World Health Organization, 2017a).

Moreover, MDD is ranked the second leading cause of years lived with disability by the World Health Organisation World Mental Health Surveys (Kessler *et al.*, 2015) and the average age of onset for MDD is between the ages of 22 and 26 years (Tomlinson *et al.*, 2009; Kessler & Bromet, 2013), however the onset of MDD can occur at nearly any age. In South Africa, a lifetime prevalence of 9.8% has been estimated for MDD (Tomlinson *et al.*, 2009), which is lower compared to the estimated 19.2% for the United States of America (Tomlinson *et al.*, 2009; Kessler & Bromet, 2013), but alarming nonetheless.

2.2.1 Epidemiology in children and adolescents

Children and adolescents are not exempt from developing MDD. MDD affects 4-8% of adolescents and nearly 2.5% of pre-adolescents (Kessler *et al.*, 2001; Bylund & Reed, 2007) and 0.3% of pre-schoolers (Kozisek *et al.*, 2008). Moreover, a meta-analysis of 41 studies conducted between the years 1985 and 2012 in 27 countries estimates a global prevalence of 1.3% for MDD in children and adolescents (Polanczyk *et al.*, 2015). Relapse proves to be a great concern, as a rate of relapse of 40% after 2 years and 70% after 5 years have been shown in children 6-12 years of age (Luby *et al.*, 2009). Worldwide, 20-25% of children between the ages of 13-18 years will experience a major depressive episode (MDE) (Rubenstein *et al.*, 2015) and it is also during this adolescent phase when young women will be more prone to develop MDD than young men (Hankin *et al.*, 1998).

Moreover, both childhood and adolescent depression predict an increased risk of relapse during adulthood (Rosso *et al.*, 2005b; Bhatia & Bhatia, 2007), however adolescent depression predicts a higher risk of relapse during adulthood compared to childhood depression (Ryan, 2005; Basu & Reddi, 2012). Paediatric MDD does not only have a high recurrence rate in adulthood, making it a predictor of long-term MDD (Pine *et al.*, 1998; Ryan, 2005), but it is also a predictor of anxiety disorders (Bufferd *et al.*, 2012; Luby *et al.*, 2014), enduring psychosocial impairment and functional impairment in adulthood (Pine *et al.*, 1998; Weir *et al.*, 2012).

Furthermore, severe and untreated MDD frequently leads to suicide, resulting in an estimated 1 million annual deaths globally (World health organization, 2012). In addition, MDD is also implicated in paediatric suicide, making suicide the fourth leading cause of death in pre-adolescents (Hulvershorn *et al.*, 2011b) and the leading cause of death in adolescents (Brown *et al.*, 2013; World Health Organization, 2017b), further highlighting the need for safe and effective treatment strategies for paediatric MDD.

2.3 Signs and symptoms

MDD is a devastating psychiatric disorder, defined as a cluster of very specific symptoms, with related impairment (Thapar *et al.*, 2012). *Anhedonia* is a common characteristic of MDD, defined as a loss of pleasure and/or interest in pleasurable activities (Bylund & Reed, 2007; Willner *et al.*, 2013). Other symptoms related to MDD include lowered mood, as well as behavioural, cognitive, psychomotor and other related dysfunctions, as listed in the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-V) (American Psychiatric Association, 2013). Moreover, individuals living with MDD may experience both physical and psychological symptoms and these symptoms may be chronic or recurrent, influencing all aspects of the affected individual's life (Kemp *et al.*, 2012). Several studies have also shown that women have a higher prevalence of *atypical* (Angst *et al.*, 2002; Lamers *et al.*, 2010), *anxiety-like* (Clayton *et al.*, 1991) and *somatic* symptoms (Silverstein, 2002) compared to men. A list of signs and symptoms of MDD follows in **Table 2-1** below.

Table 2-1: A list of signs and symptoms of MDD. Adapted from (Weissman *et al.*, 1999; Andersen & Navalta, 2004; Ryan, 2005; Bhatia & Bhatia, 2007; Bylund & Reed, 2007; NIMH, 2011; O'Donnell & Shelton, 2011; American Psychiatric Association, 2013).

Signs and symptoms of MDD
• *Anhedonia* • Anxiety • Changes in appetite, increased or decreased appetite • Changes in sleep, initial/middle/ terminal insomnia or hypersomnia • Depressed mood • Impaired attention and/or short-term memory • Impaired or irritable family and/or peer relationships • Irritability • Lethargy • Loss of social, cognitive and interpersonal skills and/or interest • Persistent feeling of emptiness, hopelessness and/or worthlessness • Physical manifestations (e.g. muscular aches and pains, headaches and digestive abnormalities) • Poor school attendance • Psychomotor agitation or retardation • Risk for self-harm • Social withdrawal • Substance abuse • Suicidal ideation and/or behaviour • Tedium • Weight gain or loss (a change of >5%)

Moreover, one study proposes that the clinical presentation and course of MDD are the same during childhood, adolescence and adulthood (Kovacs, 1996), whereas other more recent studies suggest that MDD in pre-pubertal children differs from MDD in adolescence and adulthood with respect to some causative, epidemiological and prognostic features (Weissman *et al.*, 1999; Andersen & Navalta, 2004; Bylund & Reed, 2007; Thapar *et al.*, 2012). That being said, MDD has various symptoms that can emerge in childhood, only appear in adolescence, emerge in adulthood or appear during early-life and remit (Dekker *et al.*, 2007).

Children younger than 7 years may have difficulty communicating their internal mood state and therefore MDD in this age group can differ with regards to clinical presentation (Bhatia & Bhatia, 2007), viz. vague somatic symptoms (even pain), eating disorders, anxiety, refusal to attend school and/or behavioural problems (Thapar *et al.*, 2012). Therefore, not surprisingly, the signs and symptoms of MDD are the most challenging to identify in infants and pre-schoolers, due to their inability to vocalise feelings of sadness. In this age group, symptoms of MDD must be deduced from overall changes in behaviour, including apathy, withdrawal from caregivers, delay or regression of developmental milestones and failure to flourish without any indication of a physiological cause (Son & Kirchner, 2000).

Furthermore, children are more likely to display restlessness, separation anxiety, phobias and hallucinations (Williams *et al.*, 2009; Soutullo & Figueroa-Quintana, 2013). In addition, childhood MDD may also result in personality disorders in susceptible individuals, due to the influence that MDD has on the developing personality (Weissman *et al.*, 1999; Andersen & Navalta, 2004; Bylund & Reed, 2007), whereas adolescents are more likely to experience *anhedonia*, boredom, hopelessness, hypersomnia, weight changes, alcohol and/or drug abuse and suicide attempts (Williams *et al.*, 2009; Soutullo & Figueroa-Quintana, 2013).

Moreover, paediatric MDD is also related to decreased rapid eye movement (REM) latency and REM density during sleep, hypercortisolaemia, elevated markers of inflammation, decreased neurotrophic factors (e.g. brain-derived neurotrophic factor (BDNF)) and changes in frontolimbic and frontostriatal pathways (Rao, 2013). However, children and adolescents do not present with hypercortisolaemia as frequently as adults (Braw *et al.*, 2006).

2.4 Diagnosis

As previously mentioned, the symptoms of MDD in young children and adults are comparable, with the exception that *irritability/aggression* is recognised as the primary symptom of MDD in children compared to a *depressed mood* in adults (Thapar *et al.*, 2012). Furthermore, childhood MDD often presents in conjunction with other psychological illnesses, for example anxiety disorders (Rice, 2014; Waszczuk *et al.*, 2014).

As stated by the DSM-V, MDD is diagnosed when at least one of the first two *essential* symptoms, with any four (or more) of the *additional* symptoms, as listed in **Table 2-2** below, appear for a minimum of two weeks. This must represent a change from previous functioning and cause a disturbance in the typical daily functioning of the individual (American Psychiatric Association, 2013). Moreover, it is recommended that the criteria for the diagnosis of MDD in children and adolescents, do not differ from that for adults (American Academy of Family Physicians, 2000).

For a diagnosis of MDD, adherence to the following criteria is pivotal (American Psychiatric Association, 2013):

1. At least one of the symptoms should be in the category of Essential Criteria (see **Table 2-2** below);
2. Secondly, the symptoms must cause clinically noteworthy distress for the individual or impairment in social, occupational or other vital areas of functioning;
3. The depressive episode(s) is not ascribed to symptoms of any other medical condition;
4. The occurrence of an MDE is not best described by any other psychotic disorder; and

5. There is no history of either a manic or hypomanic episode.

Table 2-2: Diagnostic criteria for the diagnosis of MDD, as set out in the DSM-V (American Psychiatric Association, 2013).

Essential Criteria	Additional Criteria
1. **Depressed mood** most of the day, nearly every day – this can appear as an *irritable mood* in children and adolescents 2. A significant **decreased interest and/or pleasure** in all/nearly all activities most of the day, nearly every day – *anhedonia*	1. Fatigue or decreased energy levels experienced nearly every day 2. Feeling worthless or unfitting feelings of guilt nearly every day 3. Insomnia or hypersomnia nearly every day 4. Psychomotor agitation or retardation nearly every day 5. Reduced ability to think and/or concentrate, or indecisiveness nearly every day 6. Repetitive thoughts of death and/or ideation of suicide 7. Significant weight loss (when not dieting) or weight gain, or a decreased or increased appetite nearly every day

From the above criteria, it is evident that the diagnosis of MDD relies on a set of mostly changeable and comparative subjective symptoms. Therefore, MDD may be seen as a heterogeneous syndrome, occurring with a variety of patterns of many distinctive symptoms (Liebenberg, 2009).

2.5 Aetiology of major depressive disorder

MDD is a complicated disorder and there is currently no agreement on a single simple or unifying hypothesis regarding the underlying neurobiological mechanism(s) involved that would explain all key aspects related to the disorder. With that said, several hypotheses for the neurobiological basis of MDD have been postulated and further refined to better our understanding of underlying neurobiological mechanism(s) involved (Belmaker & Agam, 2008). Research strongly proposes that the interplay between genetics, the neuroendocrine system and the brain is affected by both psychosocial and other environmental risk factors (Thapar *et al.*, 2012; Pryce & Klaus, 2013). That said, a *familial history* appears to be the best predictive risk factor for the development of MDD, especially in children (Nestler *et al.*, 2002; Belmaker & Agam, 2008).

A subject of heavy debate relates to whether the aetiology of MDD in children and adolescents differ from that in adulthood. Paediatric- and adult-onset MDD display differences in psychosocial risk profiles, with paediatric-onset MDD associated more significantly with family adversity, parental neglect and troublesome peer relationships compared to adult-onset MDD (Thapar *et al.*, 2012). Therefore, the aetiology of MDD in children and adolescents may differ in certain aspects (e.g. environmental triggers) from that in adults, whereas the general neurobiology seems to be comparable. As a result, present hypotheses for the neurobiological basis of MDD that are commonly associated with MDD in adults can also be associated with MDD in children, albeit with subtle modifications (Schoeman, 2015). Some hypotheses for the neurobiological basis of MDD with references are listed in **Table 2-3** below.

Table 2-3: A list of hypotheses for the neurobiological basis of MDD with references.

List of hypotheses for the neurobiological basis of MDD	References
• **Genetics** and the **gene-environment** hypothesis	(Lesch, 2004); (Ansorge *et al.*, 2007); (Heim & Binder, 2012)
• **Cholinergic supersensitivity** hypothesis of depression	(Janowsky *et al.*, 1972); (Dilsaver, 1986); (O'Leary *et al.*, 2015)
• **Monoaminergic** hypothesis of depression	(Schildkraut, 1965); (Nestler *et al.*, 2002); (Berton & Nestler, 2006a); (Haase & Brown, 2015)
• **Neuroplasticity** hypothesis	(Schmidt & Duman, 2007); (Dwivedi, 2009)
• **Glutamatergic** hypothesis	(Skolnick *et al.*, 2010); (Sanacora *et al.*, 2012)
• **Hypothalamic-pituitary-adrenal-axis hyperactivity** hypothesis	(Carroll *et al.*, 1981); (Pariante & Lightman, 2008)
• The role of the **immune system** and **inflammation**	(Dantzer *et al.*, 2008); (Merrill, 1992); (Vitkovic *et al.*, 2000)
• **Oxidative stress**	(Maes *et al.*, 2009); (Maes *et al.*, 2011a); (Leonard & Maes, 2012)
• The role of the **kynurenine pathway**	(Dantzer *et al.*, 2008); (Maes *et al.*, 2011b)
• **GABAergic deficiency** hypothesis	(Petty & Schlesser, 1981); (Cryan & Slattery, 2010); (Luscher *et al.*, 2011)

Due to their relevance to this study, only the first five hypotheses for the neurobiological basis of MDD listed in **Table 2-3** above will be discussed in section 2.5.1 below, i.e. genetics and the gene-environment hypothesis, cholinergic supersensitivity hypothesis, monoaminergic hypothesis, neuroplasticity hypothesis and glutamatergic hypothesis of depression.

2.5.1 Hypotheses for the neurobiological basis of MDD relevant to the current study

There is overall agreement that neurobiological alterations are central to MDD and that the aetiology of MDD may be best explained by a model that accounts not only for environmental factors, but also for biological (i.e. physiological and biochemical), neurodevelopmental and genetic causes. To better understand drug treatment, the neurobiological aspects are of greater importance. The numerous hypotheses for the aetiology of MDD are largely connected and several of these hypotheses (relevant to the current study) will be discussed in greater detail below, starting with the genetic risk of developing MDD and the impact of the environment on genes, followed by hypotheses that seek to explain the neurobiological basis of MDD.

2.5.1.1 Genetics and the gene-environment hypothesis

With a genetic risk of 40-70%, MDD is considered to be an extremely heritable disorder (Nestler *et al.*, 2002; Jacobson & Cryan, 2007; Belmaker & Agam, 2008). In addition to genetic susceptibility, non-genetic risk factors (remaining 30-60%) constitute the total risk for the development of MDD in children and adolescents (Nestler *et al.*, 2002; Andersen, 2003) and include environmental/psychosocial risk factors, i.e. exposure to adverse events, emotional trauma, experiences of stress, effects of some drugs, some viral infections and/or dysfunctional processes during neurodevelopment (Nestler *et al.*, 2002; Andersen, 2003). However, genetic susceptibility and environmental stressors usually co-occur in MDD and interactions between environmental factors and a genetic susceptibility to develop MDD are suggested to result in MDD (i.e. the **gene-environment hypothesis**) (Lesch, 2004), as illustrated in **Figure 2-4** below. Thus, harmful environmental influences in an already genetically vulnerable individual will increase the probability of the individual developing MDD in early-life or during adulthood (Caspi *et al.*, 2003; Ansorge *et al.*, 2007).

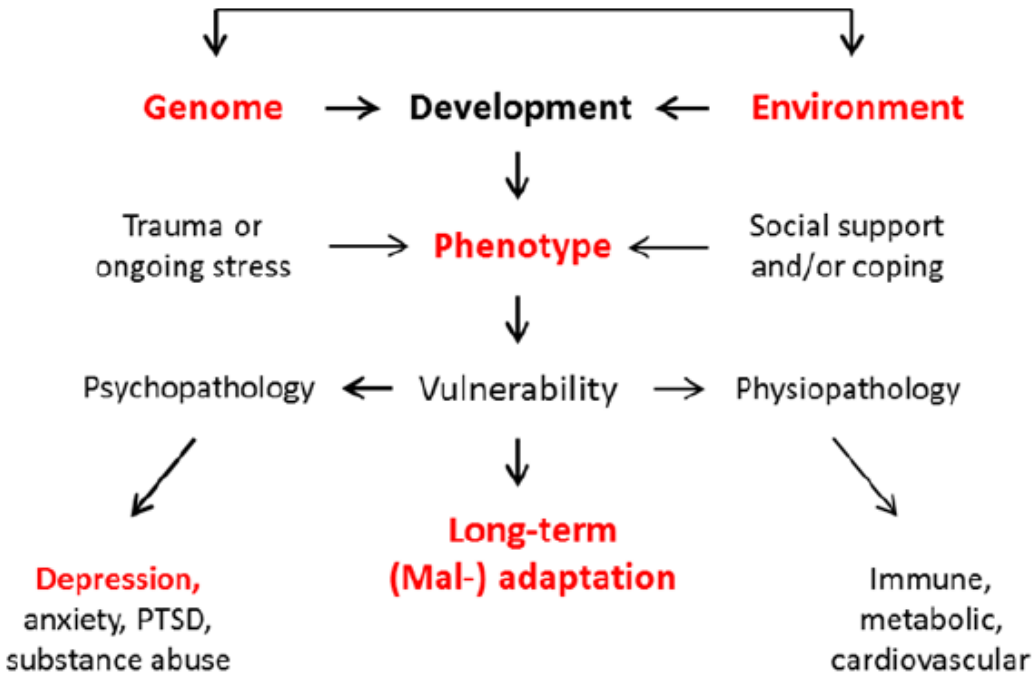

Figure 2-4: A conceptual model of the interaction between environmental factors and a genetic predisposition for developing MDD, resulting in a vulnerable phenotype. Adapted from (Heim & Nemeroff, 2001).

Environmental factors can influence the developing brain positively or negatively during specific and crucial phases of development (Andersen & Navalta, 2011). With that in mind, an unfavourable outcome is frequently only expressed in the phenotype (observable characteristics of an individual) when the environmental trigger arises in the presence of an allowing genetic profile, i.e. genetic susceptibility (Lesch, 2004; Heim *et al.*, 2008). However, not every individual that suffers from MDD has a genetic predisposition to develop the disorder, since MDD may present itself unrelated to family history (Kiyohara & Yoshimasu, 2009b).

A variety of genes that can undergo polymorphism have been identified and associated with the development of MDD. Proteins expressed by these genes are listed in **Table 2-4** below.

Table 2-4: A list of proteins expressed by genes subject to polymorphic alterations and their functions within neurobiological systems (Kiyohara & Yoshimasu, 2009b).

Proteins expressed by genes subject to polymorphic alterations	Functions within neurobiological systems
$5HT_{1A}$ receptor	• Auto-receptors in serotonergic synapses - stimulation of these auto-receptors leads to a reduction in the release of serotonin into the synapse (negative feedback mechanism) • Facilitate serotonergic activity in the cortex and limbic system
Noradrenalin (NA) transporter	Regulates the pre-synaptic reuptake of noradrenalin, as well as physiological noradrenergic effects
Serotonin transporter	Modulates serotonergic neurotransmission and removes serotonin from the extracellular space (synaptic cleft) through the presynaptic reuptake of serotonin
Tyrosine hydroxylase	The enzyme responsible for dopamine (catecholamine) synthesis
Tryptophan hydroxylase 1	The rate-limiting enzyme responsible for serotonin synthesis
BDNF	• Crucial for synaptic and neuroplasticity • Modulates neural function
Catechol-O-methyltransferase (COMT)	The enzyme responsible for catecholamine (dopamine, adrenaline and noradrenaline) metabolism

Therefore, MDD appears to be a disorder associated with complex genetic variations (Brand *et al.*, 2015) and has a high heritability rate (Belmaker & Agam, 2008), with recurrent forms of MDD more strongly related to heritability (Kendler *et al.*, 1999).

2.5.1.2 Cholinergic supersensitivity hypothesis of depression

Janowsky and colleagues postulated that there may be a cholinergic hyperactivity or a cholinergic supersensitivity in the brain of individuals suffering from MDD, with a related adrenergic underactivity (Janowsky *et al.*, 1972; Dilsaver, 1986). However, the validity of the cholinergic model of depression was questioned soon after it was first proposed, because most anticholinergic drugs failed to be effective in the treatment of MDD. Yet, it was ultimately discovered that the muscarinic receptor antagonist, scopolamine, is in fact effective in the treatment of treatment-resistant depression (Drevets & Furey, 2010; Drevets *et al.*, 2013). In addition, elevated central acetylcholine (ACh) levels have been observed in individuals suffering from MDD (Mineur *et al.*, 2013) and there are also conflicting reports suggesting that traditional antidepressants may cause an overall downregulation of central cholinergic neurotransmission

(Brink *et al.*, 2004), implicating ACh in MDD. Later, both preclinical and clinical investigations substantiated the validity of the cholinergic model of depression (Caldarone *et al.*, 2004; Rabenstein *et al.*, 2006; Andreasen *et al.*, 2009; Mineur & Picciotto, 2010; Philip *et al.*, 2010; Dagytė *et al.*, 2011; Philip *et al.*, 2012; Drevets *et al.*, 2013; Voleti *et al.*, 2013; O'Leary *et al.*, 2015). However, the cholinergic model of depression cannot fully explain the neurobiological basis of MDD, but likely plays a contributory role (Dagytė *et al.*, 2011).

MDD frequently co-occurs with cognitive deficits, including impairment of learning, memory and attention processes (McGaugh & Cahill, 1997; Hasselmo, 2006; Solé *et al.*, 2015). In this regard, it is interesting to note that the brain regions implicated in these processes, namely the frontal cortex and the hippocampus, are both regulated by the cholinergic system (McGaugh & Cahill, 1997; Hasselmo, 2006). Moreover, the hippocampus may play a pivotal part in mediating cholinergic influences on stress-related behaviour, as an elevated cholinergic tone in the hippocampus gives rise to depressive symptoms (Mineur *et al.*, 2013).

Lastly, the cholinergic model of depression is further substantiated by the FSL rat (a genetic animal model of depression - discussed in section 2.9.2), which presents with elevated cholinergic activity/sensitivity in a number of regions within the brain and also inherently displays depressive-like behaviour under normal conditions (Overstreet *et al.*, 1984; Pepe *et al.*, 1988). See Dagytė and colleagues (2011) for a full review of the cholinergic model of depression (Dagytė *et al.*, 2011).

2.5.1.3 Monoaminergic hypothesis of depression

The monoaminergic hypothesis of depression postulates that MDD results from impaired monoaminergic neurotransmission. In fact, alterations in monoaminergic neurotransmission have not only been observed in adults suffering from MDD but also in depressed juveniles (Dahlström *et al.*, 2000). This hypothesis was originally proposed as a simple monoamine deficiency hypothesis that postulates reduced levels of monoaminergic neurotransmitters in the central nervous system as the neurobiological basis of MDD (Schildkraut, 1965; Nestler *et al.*, 2002; Berton & Nestler, 2006a; Haase & Brown, 2015). Thus, altered synaptic monoaminergic neurotransmitter concentrations result in dysregulated monoaminergic neurotransmission in the brain of individuals suffering from MDD (Overstreet *et al.*, 2005). This may be a consequence of defective neurotransmitter-synapse signal transfers that may cause the inhibition of crucial signalling cascades that follow, eventually inhibiting target responses, the activation of receptors and the transcription of genes and ultimately lead to the development of symptoms and behaviours associated with MDD (Kiyohara & Yoshimasu, 2009a). These abnormalities in central serotonergic, noradrenergic and dopaminergic concentrations can develop due to

genetic mutations in key enzymes and/or receptors implicated in monoaminergic neurotransmission (see **Table 2-4**) (Kiyohara & Yoshimasu, 2009a). Monoamines (i.e. serotonin, noradrenalin and dopamine) are found throughout the entire central nervous system and modulate many areas of emotion, thought and behaviour (Belmaker & Agam, 2008; Kuramochi & Nakamura, 2009).

The discovery of monoaminergic deficiencies in the central nervous system of individuals suffering from MDD was instrumental in driving the development of antidepressants that promote serotonergic, noradrenergic and more recently dopaminergic neurotransmission in the central nervous system (Hindmarch, 2002; Booij *et al.*, 2015). In fact, most antidepressants aim to restore monoaminergic balance and functionality in the brain, by improving monoaminergic neurotransmission (Harvey & Slabbert, 2014). This is usually associated with an initial increase in synaptic monoaminergic neurotransmitter concentrations (Harvey & Slabbert, 2014) and increasing postsynaptic neuronal stimulation, ultimately alleviating the symptoms of MDD (Ansorge *et al.*, 2007; Belmaker & Agam, 2008; Mahar *et al.*, 2014b). In this regard, the mechanisms by which the monoaminergic concentrations within the synaptic cleft can be elevated include inhibiting the (Baldessarini, 1989):

- presynaptic reuptake of monoamines from the synaptic cleft;
- intraneuronal metabolism of monoamines; and
- presynaptic inhibitory auto- and hetero-receptors.

New generation antidepressants function to elevate cortical noradrenaline and dopamine concentrations and as a consequence supplement frontal cortical activity, in particular that of cognitive processing (Harvey & Slabbert, 2014). Tianeptine represents an interesting exception in that it promotes the presynaptic reuptake of serotonin via stimulation of the serotonin reuptake transporter (SERT), which would be associated with reduced levels of the monoamine in synapses, yet it still produces antidepressant effects (Mennini *et al.*, 1987; Brink *et al.*, 2006).

Nevertheless, the monoamine deficiency hypothesis does not completely explain all the features of the aetiology of MDD (Hindmarch, 2002). In fact, the monoamine deficiency hypothesis has a few shortcomings, including the observation that monoaminergic depletion in healthy individuals fails to cause MDD, whereas the depletion of monoamines induces a relapse in individuals successfully treated with SSRIs (Belmaker & Agam, 2008; Maes, 2011a), certain drugs (e.g. cocaine and amphetamines)elevate brain monoaminergic activity, but are clinically ineffective as antidepressants (Krishnan & Nestler, 2010), not all individuals that suffer from MDD respond equally to the same antidepressant and an elevation in monoaminergic concentrations at a synaptic level is noticeable within a few hours after the administration of an

antidepressant, whereas antidepressive effects are only observed after constant administration of an antidepressant for several weeks (Baldessarini, 1989). One could here also add the SERT-stimulating effects of the atypical antidepressant tianeptine, as alluded to above. All the above-mentioned further support the notion that there are additional neurochemical factors implicated in the neuropathology of MDD.

As a result, the monoamine deficiency hypothesis has been adapted in an effort to more accurately describe MDD. The adapted hypothesis proposes that the acute elevation in monoaminergic concentrations at a synaptic level may only be an early step in a possible complex cascade of events which eventually leads to antidepressive activity (Piñeyro & Blier, 1996). The delay in the onset of antidepressant action following the administration of an antidepressant has been ascribed to the desensitization of inhibitory auto- and hetero-receptors that must first take place (taking weeks) after an elevation in synaptic monoaminergic concentrations occurs (Elhwuegi, 2004). Moreover, the inhibition, desensitization or downregulation of nerve terminal auto-receptors have been shown to supplement the therapeutic response to antidepressants (Elhwuegi, 2004). This further substantiates the theory that antidepressive effects are a consequence of long-term adaptive alterations in the monoaminergic auto- and hetero-regulatory receptors (Elhwuegi, 2004).

Thus, the emphasis has not entirely been moved away from monoamines, instead the emphasis has moved towards a neural dysfunction syndrome (Groves, 2007). In this regard, a decrease in secondary messengers or in the reaction of secondary messengers to stimulation by monoaminergic neurotransmission may impair the functioning of neurotransmitters (monoamines), even without influencing the synaptic concentration of monoamines and this may account for the high occurrence of treatment failure (Groves, 2007; Belmaker & Agam, 2008). With that said, the attention has recently been redirected to the essential function of serotonin, long known to play a role in the aetiology of MDD (Limón-Morales *et al.*, 2014). Yet, the renewed focus is on the recognition of the role serotonin plays in the **susceptibility** to develop MDD and not on serotonin's primary role as a neurotransmitter (Limón-Morales *et al.*, 2014). Furthermore, there is sound evidence that serotonin also plays a pivotal part in neurodevelopment (discussed more broadly in section 2.6.3.2.1) (Whitaker-Azmitia, 2001; Fakhoury, 2016), linking serotonin to the current study.

Although studies on the role that dopamine plays in the pathophysiology of MDD have largely been overshadowed by studies on serotonin and noradrenaline, it has long been understood that dopamine plays a crucial part in various pleasurable experiences and reward (Dunlop & Nemeroff, 2007). Dopamine is found in abundant concentrations in the brain where it plays a pivotal role in the mesocorticolimbic regulation of hedonic, motivational and affective behaviours

and/or sensations (Kiyohara & Yoshimasu, 2009a), whereas impairment of these functions are frequently observed in individuals suffering from MDD (Dunlop & Nemeroff, 2007). In this regard, manic symptomatologies are associated with an elevation in synaptic dopaminergic concentrations and reduced synaptic dopaminergic concentrations are associated with MDD (Furlong *et al.*, 1999).

Figure 2-5 below illustrates monoaminergic pathways in a normal human brain.

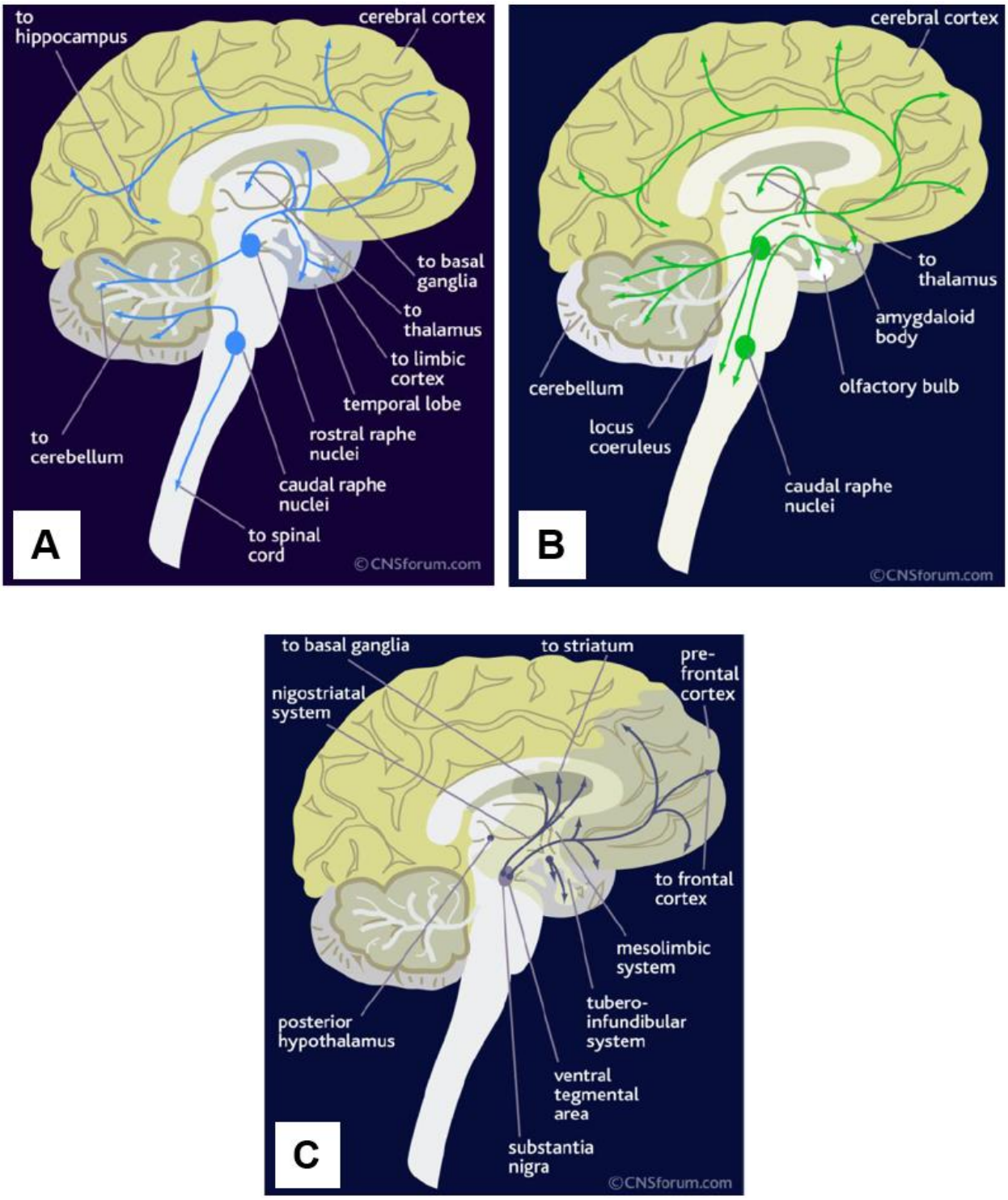

Figure 2-5: An illustration of serotonergic (A), noradrenergic (B) and dopaminergic (C) pathways in a normal human brain. Adapted from (Lundbeck Institute, 2014b).

In conclusion, it is apparent that monoaminergic neurotransmission plays an important role in the aetiology of MDD. However, shortcomings in the monoamine deficiency hypothesis emphasise the need for novel antidepressant targets in the treatment of MDD.

2.5.1.4 Neuroplasticity hypothesis

The neuroplasticity hypothesis suggests that MDD is caused by impairment of neuronal plasticity, implicating both synaptic and structural plasticity (Schmidt & Duman, 2007; Dwivedi, 2009), and has been described as a unifying hypothesis for the neurobiological basis of MDD (Schmidt & Duman, 2007; Dwivedi, 2009). According to this hypothesis, the inability of an individual to properly adapt to environmental stressors eventually causes the development of MDD and this is illustrated in **Figure 2-6** below (Schmidt & Duman, 2007; Dwivedi, 2009).

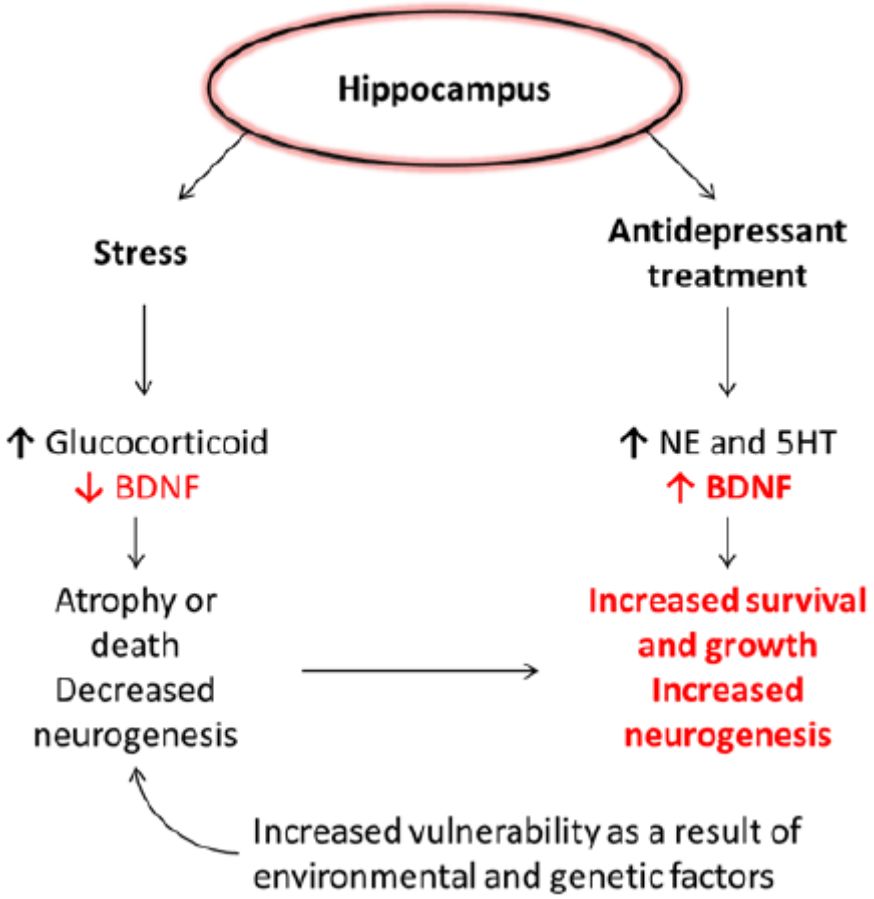

Figure 2-6: A generalised diagrammatic illustration of the effects that stress and glucocorticoids (cortisol) have on the hippocampus, mainly through a reduction in the expression of BDNF and the manner in which this is opposed by antidepressant treatment. Individual susceptibility to MDD may be the result of genetic and/or environmental factors. Adapted from (Duman *et al.*, 1999).

The neuroplasticity hypothesis is supported by profuse evidence of both synaptic and structural plasticity alterations found post mortem in individuals that suffered from MDD, especially in the hippocampus of suicidal individuals (Mahar *et al.*, 2014a), and also in rodents after subjection to stress (Bjørnebekk *et al.*, 2010). As previously mentioned, the hippocampus regulates emotion, learning and memory and is a pivotal region in the brain implicated in MDD and neuroplasticity

(Groves, 2007). With that said, neuroplasticity plays a pivotal part in the functioning of the central nervous system and enables it to receive new information, learn new skills, recognize neuronal pathways and recover from brain injuries (Gomes da Silva *et al.*, 2012). In addition, the hippocampus is an extremely stress-sensitive region in the brain, particularly during early-life neurodevelopment (Duman *et al.*, 1999) and chronic stress during adulthood has been shown to result in hippocampal atrophy (Hindmarch, 2002; Groves, 2007).

Moreover, a reduction in hippocampal volume and thickness have also been observed in juveniles suffering from MDD, which is in line with that seen in adults with the same disorder (Peterson *et al.*, 2009; Rao *et al.*, 2010) and this suggests impairment of neuroplasticity and/or an elevation in neurotoxicity. In addition, a reduction in the activation of pivotal brain regions during task-based functional neuroimaging and a reduction in cognitive functioning during cognitive control tasks are observed in depressed juveniles (Hulvershorn *et al.*, 2011a), substantiating the involvement of decreased neuroplasticity and altered glutamatergic neurotransmission in the development of juvenile MDD (Hulvershorn *et al.*, 2011a).

Several neurotrophic factors (e.g. BDNF and cyclic adenosine monophosphate response element binding protein (CREB)) play a pivotal part in hippocampal neurogenesis through cellular proliferation, maintenance and migration during neurodevelopment (Berton & Nestler, 2006a). Moreover, neurotrophic factors are partly responsible for neuroplasticity, synaptic plasticity and the survival of neurons during neurodevelopment and as a consequence, any reduction in neurotrophic factor concentrations can cause structural damage and/or a reduction in neurogenesis in the hippocampus (see **Figure 2-6**) (Berton & Nestler, 2006a). However, neurotrophic factors do not only play a fundamental role during neurodevelopment, but also in adulthood, where neurotrophic factors are required for the maintenance of neuronal functioning, neurogenesis and the structural integrity of neurons (Dwivedi, 2009). Furthermore, it has been shown that a reduction in the concentration of neurotrophic factors causes structural abnormalities in the brain and a reduction in neuroplasticity, resulting in the inability of an individual to adapt appropriately during stressful events and ultimately leads to the development of MDD (Dwivedi, 2009).

Being a secretory protein, BDNF is reduced in the hippocampus and serum, but elevated in the nucleus accumbens and nucleus accumbens-ventral tegmental area pathway of individuals suffering from MDD and chronic stress disorders (Schecterson *et al.*, 2012). Moreover, BDNF has been studied more extensively than any of the other neurotrophic factors due to its involvement in the modulation of depressive-like behaviour (Mahar *et al.*, 2014a). For instance, antidepressive-like effects have been observed in behavioural animal models of MDD following local BDNF infusions into specific brain regions (i.e. the hippocampus, midbrain or lateral

ventricles), as demonstrated by a reduction in immobility in the FST (Siuciak *et al.*, 1997; Shirayama *et al.*, 2002; Hoshaw *et al.*, 2005).

The down-regulation of BDNF expression observed in MDD has been ascribed to the effects of *pro-inflammatory cytokines* (Barrientos *et al.*, 2003), *glucocorticoids* (Barbany & Persson, 1992; Schaaf *et al.*, 1998) and a *decrease in* $5HT_{2A}$ *receptor stimulation* (Vaidya *et al.*, 1997). However, MDD presents with abnormal regulation of several neurotrophic factors in addition to BDNF, e.g. insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Fakhoury, 2016). In this regard, VEGF has been shown to enhance neurogenesis in the hippocampus, whereas a reduction in VEGF expression may lead to neuroplastic deficiencies related to MDD (Palmer *et al.*, 2000).

The part that monoamines play in neuroplasticity (with reference to MDD) has also been studied and it has been shown that an elevation in synaptic monoaminergic concentrations causes an increase in neurotrophic factor release, with a subsequent elevation in hippocampal synaptic plasticity and neurogenesis (Haase & Brown, 2015). Importantly, the glutamate–nitric oxide–cyclic guanosine monophosphate-protein kinase G (Glu-NO-cGMP-PK-G) pathway is also implicated in *neuroplasticity* (Zarate *et al.*, 2003; Calabrese *et al.*, 2007; Kleppisch & Feil, 2009), *MDD* (Zarate *et al.*, 2002; Volke *et al.*, 2003a; Zarate *et al.*, 2003; Sanacora *et al.*, 2008) and *anxiety disorders* (Eroglu & Caglayan, 1997; Volke *et al.*, 1997; Volke *et al.*, 2003a; Volke *et al.*, 2003b) and since the Glu-NO-cGMP-PK-G pathway is the main focus of this study, a broader discussion of this pathway can be found in section 2.8.

Furthermore, the administration of antidepressants leads to an increase in neurogenesis, synaptogenesis and the maturation of neurons, corresponding with an elevated BDNF expression (Duman *et al.*, 1999; Kozisek *et al.*, 2008). Therefore, antidepressant treatment normalises the reduced BDNF concentrations observed in individuals suffering from MDD (Hasselbalch *et al.*, 2012). In fact, several classes of antidepressants have been proven to be effective in reversing neuroplasticity deficits observed in individuals suffering from MDD through stimulating neuro- and gliogenesis, promoting cellular endurance, enhancing synaptic birth and increasing dendrite branching (Serafini, 2012). Moreover, SSRIs depend on BDNF signalling to promote neurogenesis (with a subsequent reduction in hippocampal atrophy) and to bring about an antidepressive-like effect (Haase & Brown, 2015), suggesting a link between MDD, BDNF and serotonin (Haase & Brown, 2015).

However, the dependency on neurogenesis for antidepressive-like effects is not exclusive to SSRIs (Zhao *et al.*, 2008), as the antidepressive action of nearly all antidepressant treatments is prevented by the inhibition of neurogenesis (Sahay & Hen, 2007; Pittenger & Duman, 2008).

Therefore, it is proposed that neurogenesis may be central to the ability of the brain to adjust to new circumstances and when neurogenesis is compromised, maladaptive learning responses occur (Krishnan & Nestler, 2008). However, a reduction in neurogenesis per se does not cause MDD, as the inhibition of neurogenesis does not induce depressive-like behaviour in rodents (Santarelli *et al.*, 2003; Surget *et al.*, 2008). Finally, the antidepressant-related (e.g. MOAI, TCA, SSRI, ECT and ketamine) reversal of neuroplasticity deficits is further substantiated by reviews conducted on studies involving both humans and relevant animal models of MDD (Pittenger & Duman, 2008; Serafini, 2012).

2.5.1.5 Glutamatergic hypothesis

The foundation for this hypothesis was the discovery that NMDA receptor antagonists give rise to antidepressive-like effects (Skolnick *et al.*, 2010) and the glutamatergic hypothesis is associated with the neuroplasticity hypothesis of MDD (see section 2.5.1.4), regarding the pathophysiological effects on neuroplasticity, synaptic plasticity, neurogenesis and neuronal circuitry (Sanacora *et al.*, 2012). Also, clinical evidence support glutamatergic abnormalities as an underlying cause for the development of MDD, with associated cognitive dysfunction (Sanacora *et al.*, 2012). In this regard, variations in glutamatergic concentrations were observed in individuals suffering from MDD, as measured in the brain, cerebrospinal fluid and plasma (Sanacora *et al.*, 2012). Moreover, a study conducted on individuals suffering from MDD reported the following conclusions: an elevated central glutamatergic concentration, a reduced glutamine-to-glutamate plasma ratio and an increased platelet glutamatergic concentration (Hasler *et al.*, 2007). In addition, elevated glutamatergic concentrations were also found in post-mortem studies on the frontal cortices of individuals that suffered from MDD (Hashimoto *et al.*, 2007).

Excessive glutamatergic secretion may be the result of profound stress stimuli and results in subsequent alterations in synaptic neurotransmission, decreased synaptic spine formation, dendrite malformation and loss of glial cells (McEwen, 2005; Pittenger & Duman, 2008). Moreover, the detrimental effects of an excess in synaptic glutamate on surrounding tissues have been documented, including neurodegeneration, neurotoxicity and a reduction in neuroplasticity (Koolschijn *et al.*, 2009). Yet, both an increase and a decrease in glutamatergic metabolite concentrations have been noted in a variety of brain regions of individuals suffering from MDD (Hasler *et al.*, 2007; Sanacora *et al.*, 2012).

Antidepressant treatment reduced plasma glutamatergic measurements in one study (Hasler *et al.*, 2007) and clinical studies have demonstrated that the prevention of presynaptic glutamatergic release alleviates the symptoms of MDD (Calabrese *et al.*, 1999; Zarate Jr *et al.*,

2004). In addition, the potentiation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor function has been shown to have antidepressive-like effects in preclinical models of MDD (Li *et al.*, 2001; Black, 2005), with an associated enhancement of neurogenesis (Bai *et al.*, 2003) and an elevation in the expression of neurotrophic factors (Lauterborn *et al.*, 2000; Lauterborn *et al.*, 2003). Not surprisingly, AMPA receptors have been associated with learning and memory processes (Sanderson *et al.*, 2008). Furthermore, preclinical studies propose that agonists at particular subtypes of G protein-coupled metabotropic glutamate receptors (mGluR) give rise to antidepressive-like, anxiolytic-like and neuroprotective effects (Maiese *et al.*, 2000; Pałucha *et al.*, 2004).

Thus, glutamate and its receptors are implicated in the neurobiology of MDD. However, there are several challenges in actualizing clinically effective antidepressants that modulate glutamatergic neurotransmission, for instance the enhancement of glutamatergic neurotransmission causes excitotoxicity and neuronal cell death (Frandsen *et al.*, 1989), whereas glutamatergic receptor antagonists have problematic neuropsychiatric side effects (Riederer *et al.*, 1991). An illustration of the glutamatergic pathways in a normal human brain follows in **Figure 2-7** below.

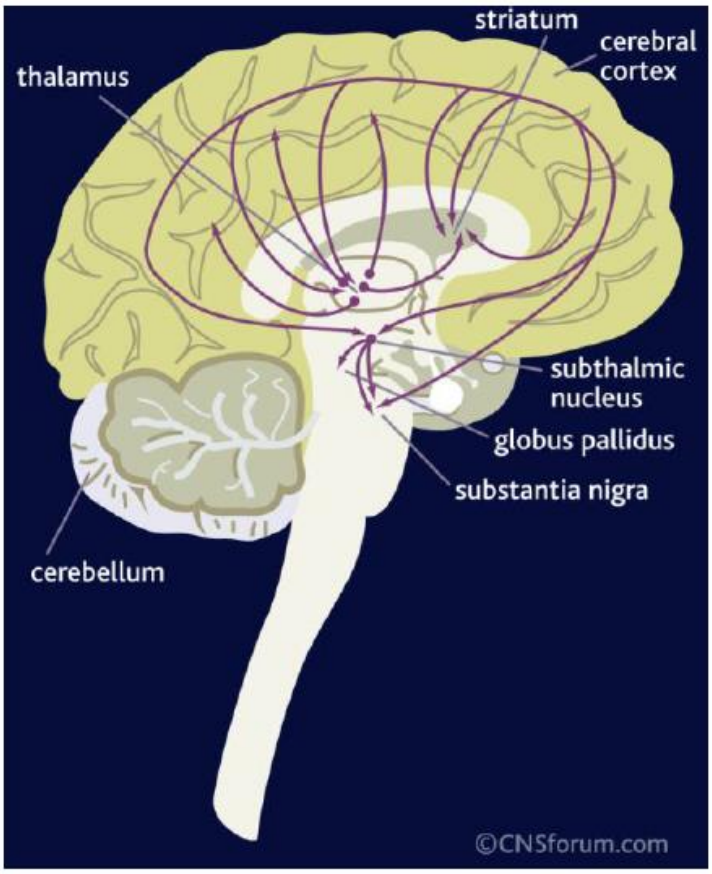

Figure 2-7: An illustration of glutamatergic circuits in a normal human brain (Lundbeck Institute, 2014a).

The precise mechanisms that underlie the glutamatergic hypothesis of MDD are unclear and more research is needed to shed light on glutamate's involvement in the pathophysiology of MDD before any conclusions can be drawn.

2.6 Neurobiology

2.6.1 Brain regions implicated in MDD

A number of brain regions have been associated with the development, manifestation and prognosis of MDD and regions identified to play a pivotal role are the prefrontal cortex, hippocampus and amygdala, as illustrated in **Figure 2-8** below. The neuropathophysiology of MDD has been studied most extensively in adults, so that much of our current understanding thereof is the result of data obtained from the adult brain. Nevertheless, some neurobiological studies have proposed that the brain regions implicated in paediatric MDD are comparable to the brain regions affected in adults suffering from the same disorder (Kowatch *et al.*, 1999; Andersen & Navalta, 2004).

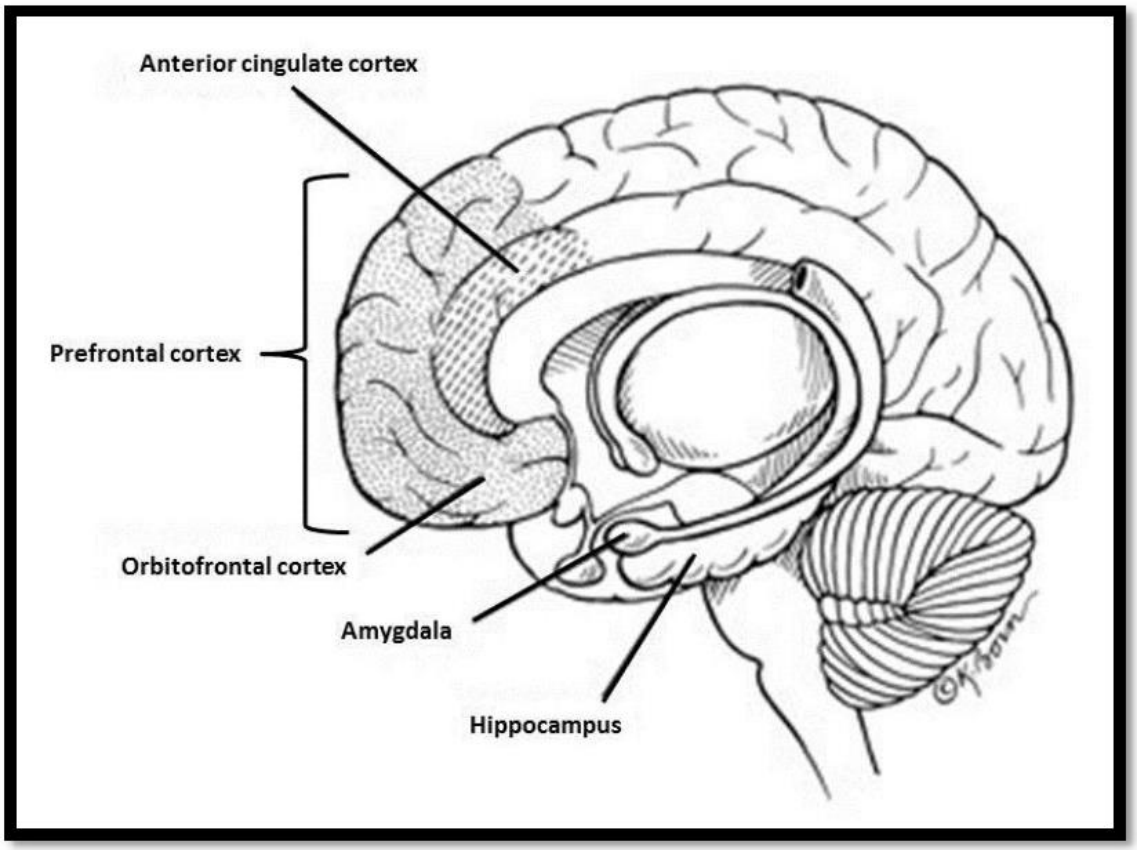

Figure 2-8: An illustration of the three major regions in the brain associated with MDD, viz. the prefrontal cortex, hippocampus and amygdala (Dana, 2011).

The frontal cortex and hippocampus are involved in the psychopathological and somatic symptoms of MDD respectively, whereas both of these brain regions are implicated in the cognitive symptoms of the disorder (McLeod *et al.*, 2001; Frodl *et al.*, 2006; Belzung *et al.*, 2015). Moreover, the frontal cortical and hippocampal regions are centrally located in the limbic-cortical network (McLeod *et al.*, 2001) and both of these brain regions are main targets of stress hormones implicated in the pathophysiology of MDD (McLeod *et al.*, 2001; Frodl *et al.*, 2002; MacQueen *et al.*, 2003a; Caetano *et al.*, 2004; Diamond *et al.*, 2004), making the frontal cortex and hippocampus suitable brain regions for studying the effects of both nitric oxide (NO) and antidepressants on mood and antidepressant action (Contestabile *et al.*, 2003). Also, NO is implicated in the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway and this neurological pathway forms the basis of the current study. Furthermore, NO plays an important part in *cortical perfusion*, learning, memory and *neuroplasticity* - all pivotal processes implicated in MDD (Contestabile *et al.*, 2003).

Studies have shown brain atrophy and neurodegeneration in patients suffering from MDD, including structural brain alterations (Kemp *et al.*, 2012; Solé *et al.*, 2015), and brain atrophy and neurodegeneration may in turn cause neurocognitive insufficiencies, viz. impaired memory, indecisiveness and loss of concentration (Kemp *et al.*, 2012; Solé *et al.*, 2015). There are pivotal neurophysiological and neuroanatomical alterations that occur in brain regions affected by MDD (i.e. the prefrontal cortex, hippocampus and amygdala) and these alterations will be discussed in more detail below.

2.6.1.1 The prefrontal cortex

The prefrontal cortex is implicated in cognitive functions (Duman *et al.*, 1999), i.e. decision making and experiencing cognitive emotions, especially optimistic (left frontal cortex) and pessimistic feelings (right frontal cortex) (Dubac, 2002). During adolescence the prefrontal cortex undergoes the most fundamental and prolonged changes compared to other regions of the brain, such as the primary motor and sensory cortices (Huttenlocher, 1979b; Bourgeois *et al.*, 1994). Changes in the prefrontal cortex of juveniles suffering from MDD include pruning of synapses (Giedd *et al.*, 1999), a reduction in frontal white matter, an increase in frontal grey matter (Steingard *et al.*, 2000) and an enlargement of the left-sided prefrontal cortex (Steingard *et al.*, 2000). Also, a reduction in regional cerebral blood flow has been shown in the left anterofrontal lobe of MDD patients (Tutus *et al.*, 1998). Moreover, these changes in the prefrontal cortex that occur in children and adolescents suffering from MDD are comparable to the changes that are seen in adults suffering from the same disorder (Andersen & Navalta, 2004).

Furthermore, individuals suffering from MDD, with a vulnerability to stress, express stress-induced histological alterations in many regions of the brain, including a reduction in the number of prefronto-cortical neuronal spines (see **Figure 2-9**), as well as a reduction in the number, length and functionality of prefronto-cortical dendrites (see **Figure 2-9**), which in turn contribute to the anhedonic features of MDD and an elevated risk of developing addictive-like behaviours (Russo & Nestler, 2013).

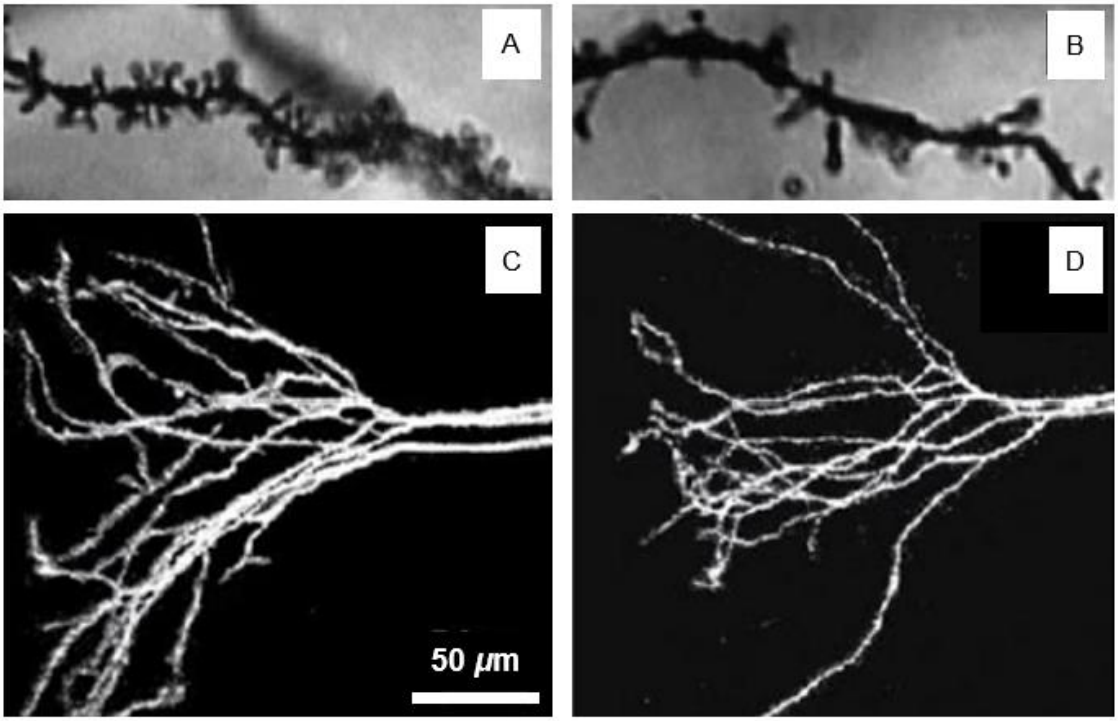

Figure 2-9: A reduction in the medial prefronto-cortical spine count as displayed in a rodent model exposed to a chronic stress paradigm (B) compared to healthy controls (A) and a reduction in the volume and length of apical dendrites in the prefrontal cortex of a rodent model exposed to a chronic stress paradigm (D) compared to healthy controls (C), with relevance to MDD. Adapted from (Pittenger & Duman, 2008; Duman, 2009).

Other structural alterations include a reduction in the number of excitatory synapses and associated gene expressions in both the prefrontal cortex and hippocampus, as well as a reduction in the width and thickness of cells in the prefrontal cortex (Rajkowska *et al.*, 1999). The alterations in the above-mentioned regions of the brain may persist long after depressive-like symptoms have subsided (Solé *et al.*, 2015).

2.6.1.2 The hippocampus

The hippocampus plays a pivotal part in long-term learning, memory and neuroendocrine stress hormone regulation (Reiman, 1997; Sapolsky, 2001a; Kim & Diamond, 2002). Moreover, a number of early studies suggest a smaller left hippocampal size in patients suffering from MDD

compared to healthy controls (Bremner *et al.*, 2000; Frodl *et al.*, 2003; MacQueen *et al.*, 2003a; MacMaster & Kusumakar, 2004), whereas other studies suggest no volume changes (Ashtari *et al.*, 1999; Bookheimer *et al.*, 2000; Vakili *et al.*, 2000). However, more advanced technology and measuring techniques (e.g. magnetic resonance imaging) made it possible to thereafter demonstrate more clearly a reduction in the left hippocampal size of patients with a long history of severe MDD (Campbell *et al.*, 2004; Videbech & Ravnkilde, 2004; Frodl *et al.*, 2008). In fact, as much as a 4-5% reduction in left hippocampal volume has been observed in patients suffering from MDD compared to healthy controls (Campbell *et al.*, 2004; Videbech & Ravnkilde, 2004; Frodl *et al.*, 2008). Furthermore, repeated MDEs result in cumulative hippocampal atrophy, which can generally be reversed by antidepressant treatment, whereas hippocampal atrophy can be permanent in individuals suffering from refractory depression (McEwen *et al.*, 1997; McEwen, 1999; Sapolsky, 2001b; MacQueen *et al.*, 2003b).

The hippocampus is further implicated in the regulation of the hypothalamic-pituitary-adrenal axis (HPA axis) stress response (Pittenger & Duman, 2008). Therefore, structural and/or neurochemical alterations in the hippocampus may cause impaired stress response coordination (Pittenger & Duman, 2008). Moreover, studies have shown that chronic stress related to MDD negatively affects hippocampal plasticity, neurogenesis and synaptogenesis (Woolley *et al.*, 1990; Reagan & McEwen, 1997), as chronic stress (leading to long-term elevated circulatory cortisol levels) is implicated in dendritic remodelling of synaptic terminal structures (Sapolsky *et al.*, 1985; Uno *et al.*, 1989; Sapolsky *et al.*, 1990; Sousa *et al.*, 2000), resulting in neuronal cell death (Sousa & Almeida, 2002; Harlan *et al.*, 2006; Czéh & Lucassen, 2007).

Children with a family history of MDD have also been shown to have a decreased hippocampal volume and this suggests a higher risk for developing MDD in later life, as posed by these detrimental morphological changes already present at a very young age (MacMaster *et al.*, 2008). With that said, constant hippocampal volume reductions are associated with a decrease in hippocampal function, leading to cognitive deficits (MacQueen *et al.*, 2003a; Frodl *et al.*, 2006) and a loss of neuroendocrine regulatory control, with subsequent increased cortisol-mediated hippocampal damage (Sapolsky, 1996).

To summarise, MDD is related to impaired hippocampal function and a reduction in hippocampal volume, in both adults and in juveniles.

2.6.1.3 The amygdala

The amygdala is located deep within the anterior, inferior temporal lobes (see **Figure 2-8**) and plays a pivotal part in certain processes, including fear, the perception of emotional stimuli

(Aggleton, 1993), psycho-social behaviours and direct emotional responses (Baxter & Murray, 2002; Drevets, 2003). A reduced amygdala volume can be seen in individuals suffering from MDD (Altshuler *et al.*, 1998; Sheline *et al.*, 1998; Strakowski *et al.*, 1999; Altshuler *et al.*, 2000; Mervaala *et al.*, 2000; Van Elst *et al.*, 2000; Hamilton *et al.*, 2008) and the amygdala core (comprising of the amygdala basal nucleus, accessory basal nucleus and lateral nucleus) was found to have a smaller volume in female patients suffering from MDD compared to males with the same disorder (Sheline *et al.*, 1998). However, a smaller right amygdala volume is seen in both male and female patients suffering from MDD, as compared to healthy controls (Mervaala *et al.*, 2000). Finally, a reduced amygdala volume can also be seen in paediatric patients suffering from MDD (Rosso *et al.*, 2005a).

2.6.2 Prefronto-cortical and -hippocampal pathways associated with MDD

Three main pathways are implicated in the top-down control over limbic responses, viz. the fronto-amygdala, fronto-striatal and fronto-hippocampal pathways (Dobson, 2008). The pathogenesis of MDD can be described as an emotional regulatory abnormality, resulting from a combination of pathogenic processes and implicates over-responsive limbic structures to stress, as well as a shortage in fronto-limbic pathways that mediate the inhibitory regulation of limbic responses (Dobson, 2008). **Figure 2-10** illustrates the involvement of the prefronto-cortical and -hippocampal pathways in MDD.

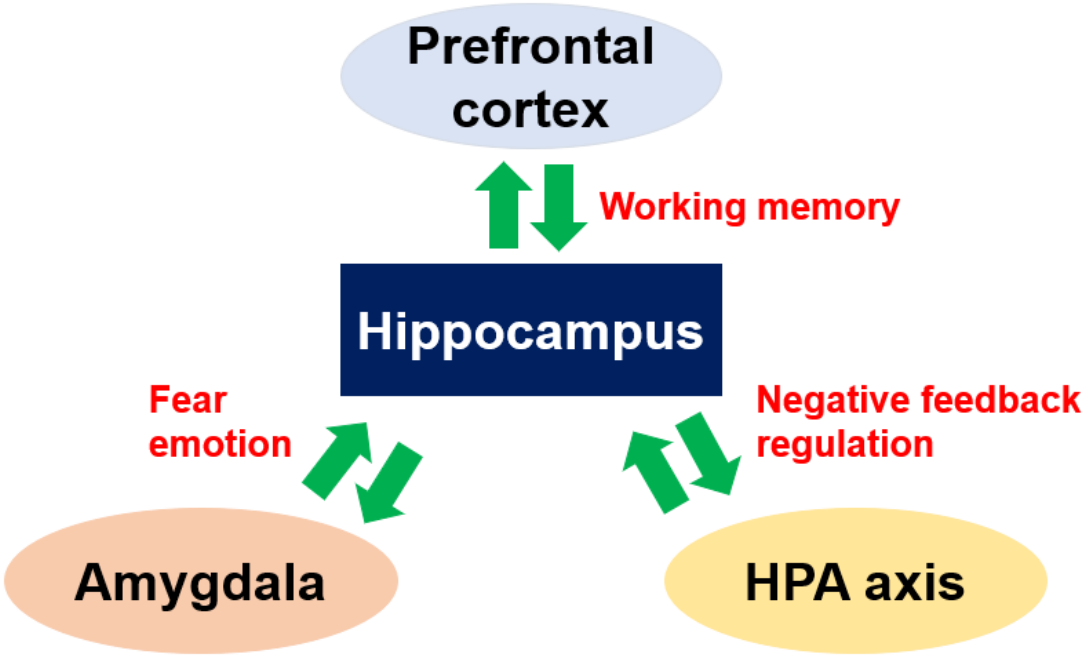

Figure 2-10: **The prefronto-cortical and -hippocampal pathways implicated in MDD** (Dobson, 2008).

The pathway between the hippocampus and the prefrontal cortex, as well as the pathway between the hippocampus and the amygdala (see **Figure 2-10**), are implicated in context regulation of affect. Also, the negative feedback mechanism of the HPA axis may be disrupted by hippocampal dysfunction, as is shown in clinical studies (Jacobson & Sapolsky, 1991; Johnstone *et al.*, 2007). Furthermore, the persistence of deficits in executive functioning experienced by individuals with remitted MDD have been associated with abnormalities in both prefrontal and hippocampal pathways, which may represent an underlying cognitive susceptibility for relapse in MDD (Clark *et al.*, 2005; Dobson, 2008).

2.6.3 Neurodevelopment

MDD is also considered a neurodevelopmental disorder that is caused by an interplay between both genetic and environmental factors (Kessler *et al.*, 2001) and these factors affect the maturation of pathways within the brain that are implicated in affective functioning, eventually leading to depressive disorders in adulthood (Ansorge *et al.*, 2007). Moreover, the rate of maturation is different for distinct monoaminergic pathways, for example the serotonergic system develops earlier than both the noradrenergic and dopaminergic systems. This phenomenon may explain the effectiveness of SSRIs in children, whereas other classes of antidepressants seem ineffective in the same age group (Murrin *et al.*, 2007). Importantly, the developing brain is constantly changing and adapting, making it more vulnerable to external influences compared to the adult brain (Andersen & Navalta, 2004).

Neurodevelopment is an extremely intricate process and the functional integrity of the brain during adulthood can be determined by various stimuli during this period (Gomes da Silva *et al.*, 2012). For instance, pre-frontal cortex functioning during adulthood can be affected significantly by social isolation during childhood and/or adolescence, resulting from an impediment of synaptic plasticity and a reduction in both serotonergic and dopaminergic neurotransmission (Baarendse *et al.*, 2013). In addition, hippocampal functioning during adulthood (i.e. learning, memory and various emotional processes) is also adversely affected by early-life stressful stimuli and the hippocampus is extremely susceptible to neurodegenerative disorders, including MDD (Baarendse *et al.*, 2013).

Prenatal development in humans spans 36 to 40 weeks and is divided into three trimesters (12 weeks each) (Murrin *et al.*, 2007), whereas prenatal development in rats traverses 21 days and correlates neurologically to the first and second trimester in humans (see **Figure 2-11**) (Eiland & Romeo, 2013). Moreover, the neonatal period in rats (birth to weaning) correlates neurologically to the third trimester in human development (Eiland & Romeo, 2013) and is known as a protracted period of parental care following birth, spanning 14 to 21 days (Eiland &

Romeo, 2013). Rats reach sexual maturity (puberty) at around the age of 35 days. correlating neurologically to puberty or early adolescence in humans, i.e. 12 years of age (Murrin *et al.*, 2007; Eiland & Romeo, 2013). Although variations may occur, rats between the ages of 30 and 60 days undergo behavioural and neurobiological changes similar to those observed during adolescence in humans and rats reach adulthood at PnD 60 (Eiland & Romeo, 2013).

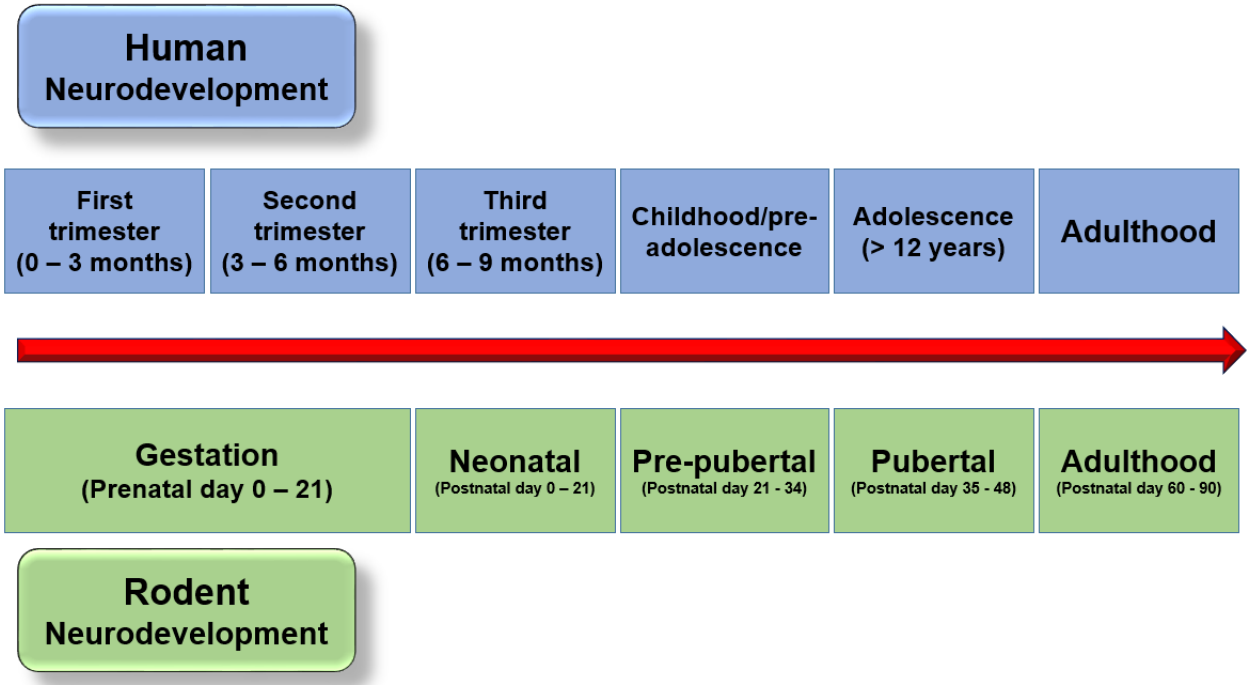

Figure 2-11: An illustration of neurodevelopment in humans versus rats. Adapted from (Kepser & Homberg, 2015).

Neurodevelopment displays surprising similarities and alignment of age-related brain development between humans and rats and considering that adolescence is an important marker for specific hallmarks in neurodevelopment, these comparative ages between humans and rats are pivotal when interpreting data from animal studies (Murrin *et al.*, 2007).

2.6.3.1 Development of the brain

Initially the brain "overdevelops", followed by synaptic "pruning" and neuronal maturation (i.e. via homeostatic processes) to adjust and refine for what is needed for optimal functioning during adulthood. With the pruning process unused cells are continuously being removed, which in part is regulated by serotonin (Whitaker-Azmitia, 2001). During adolescent development, substantial remodelling of the brain occurs, especially within limbic and cortical regions of the brain, and volumetric elevations in the hippocampus and amygdala have been shown during early puberty (Eiland & Romeo, 2013). In addition, dynamic alterations in cortical grey- and white-matter volumes occur throughout adolescence, whereas elevations in the volume of both

frontal and temporal cortices occur during childhood up until puberty, followed by cortical thinning into adulthood (Eiland & Romeo, 2013). The aforementioned neurodevelopmental changes result in differential stages of vulnerability in a regionally specific fashion (Andersen, 2003).

Moreover, both structural and functional alterations occur in the brain over the entire course of an individual's life, including alterations in neuroplasticity (i.e. the ability of the brain to change structure and function as compensatory response to environmental demands and challenges) (Andersen, 2003). As mentioned before, neuroplasticity (see section 2.5.1.4) is regulated by neurotrophic factors (growth factors) during the entire course of an individual's life and plays a pivotal part in learning and neuronal repair (Andersen & Navalta, 2004). Moreover, neurotrophic factors will continue to play an integral part in dendritic branching and the guiding of neuronal innervations during adulthood (Andersen & Navalta, 2004).

In rats, the expression of neurotrophic factors in the brain reaches a peak during the prenatal period (as neurons form their first synaptic contacts) and growth factors increase again during postnatal development, but in a region-specific manner (Andersen, 2003). Moreover, BDNF messenger ribonucleic acid (mRNA) in the hippocampus reaches a level comparable to that seen in adulthood on PnD 7 and stays elevated during early-life development, whereas cortical concentrations peak at PnD 14 and gradually decline (Andersen, 2003).

2.6.3.2 Neurotransmitters implicated in neurodevelopment and MDD

As alluded to earlier, there are three main monoaminergic systems implicated in the pathophysiology of MDD, namely the serotonergic, noradrenergic and dopaminergic systems and the stage of maturation of each of these systems remarkably affect the efficacy of antidepressant drugs administered at different developmental phases (Schoeman, 2015). **Figure 2-12** illustrates age-related neurodevelopment in rats (Schoeman, 2015). Important to note, the serotonergic system starts developing and maturing much earlier than the noradrenergic and dopaminergic systems.

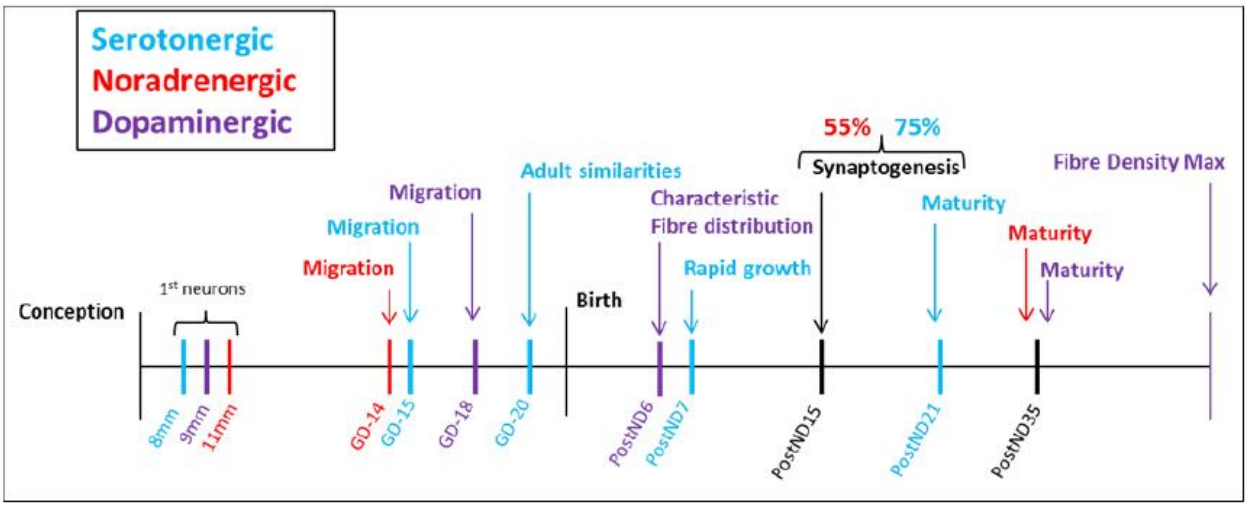

Figure 2-12: An illustration of age-related neurodevelopment in rats (Badenhorst, 2014). With abbreviations: GD = gestational day and PostND = postnatal day.

The pattern of the development of serotonergic neurons is different from that of noradrenergic neurons (see **Figure 2-12**), as the serotonergic system reaches maturity at PnD 21 (pre-puberty) and the noradrenergic system continues developing throughout pre-pubertal development, only reaching maturity at PnD 35 (puberty) (Murrin *et al.*, 2007). Therefore, the development of the serotonergic system takes place mainly during the prenatal developmental phase, whereas the development of the noradrenergic system takes place mainly during the pre-pubertal developmental phase (Murrin *et al.*, 2007). Importantly, humans display a similar age-related neurodevelopment to that seen in rats (Murrin *et al.*, 2007).

Neurotransmitters can be classified as amino acid neurotransmitters (with both ionotropic and metabotropic receptor activity), biogenic amine neurotransmitters (with metabotropic receptor activity) or gaseous neurotransmitters (e.g. nitric oxide) (Leonard, 2003). **Figure 2-13** below illustrates the classification of neurotransmitters involved in synaptic neurotransmission.

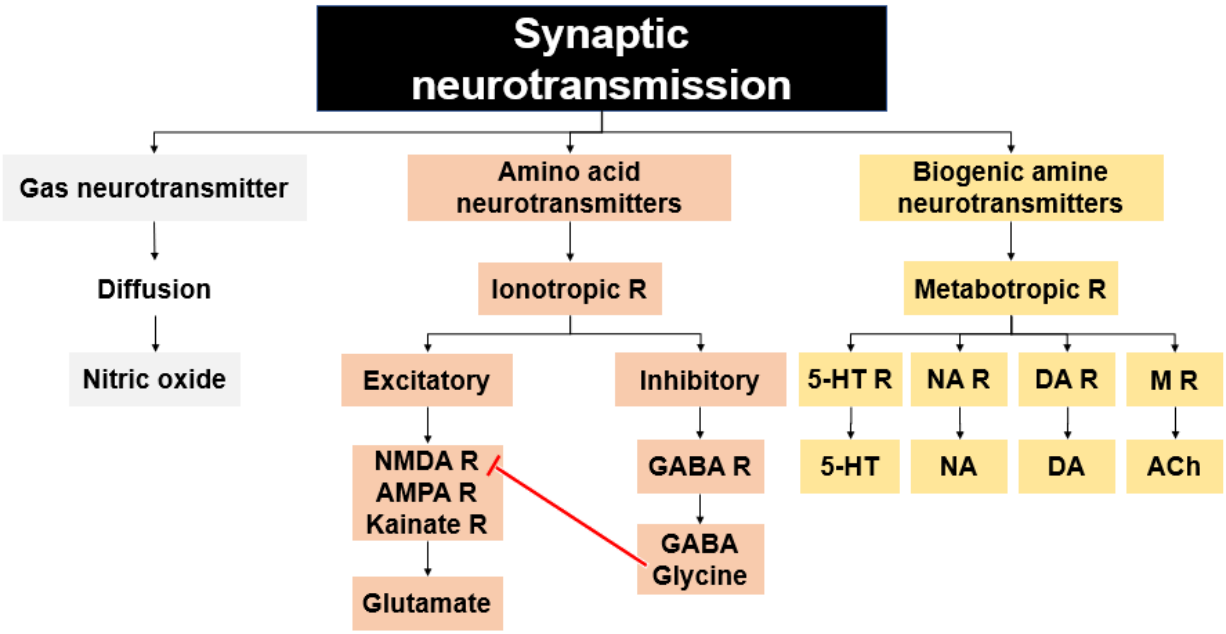

Figure 2-13: A classification of neurotransmitters involved in synaptic neurotransmission, in accordance with receptor function. Adapted from (Leonard, 2003). With abbreviations: R = receptors, NMDA = N-methyl-D-aspartate, AMPA = alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid, GABA = gamma-aminobutyric acid, 5-HT = serotonin, NA = noradrenaline, DA = dopamine, MR = muscarinic receptor and Ach = acetylcholine.

2.6.3.2.1 Serotonergic development

Dietary intake of tryptophan (amino acid) is necessary for serotonin synthesis (Kepser & Homberg, 2015). During the early stages of prenatal development, the placenta secretes and provides the foetus with serotonin and later provides the foetus with tryptophan when the foetus is able to synthesise serotonin by itself (Maes *et al.*, 2011b). Thus, tryptophan ultimately crosses the blood brain barrier and is subsequently incorporated into serotonergic neurons of the foetus (Maes *et al.*, 2011b). As one of the first neurotransmitters found in the mammalian brain (see **Figure 2-12**), serotonin is first implicated in the regulation of neurodevelopment, followed by its role as a neurotransmitter in the central nervous system (Mazer *et al.*, 1997). Considering the important role that serotonin plays in neuroplasticity, any changes in the serotonergic system during neurodevelopment may alter the normal development of the central nervous system, resulting in long-lasting effects (Kepser & Homberg, 2015).

Interestingly, the 8 mm rat embryo has been shown to already have serotonergic neurons (see **Figure 2-12**) (Murrin *et al.*, 2007) and serotonin is noticeable in rats from as early as gestational day 13, whereas serotonin is first noticeable from the fifth gestational week in humans (Murrin *et al.*, 2007). In addition, serotonergic neurons have been formed by gestational day 14 in rats and these neurons grow towards the prefrontal cortex, hippocampus and amygdala (Murrin *et*

al., 2007), whereas serotonergic neurons are spread in groups throughout the brain by gestational day 19, comparable to that observed during adulthood (Murrin *et al.*, 2007). Moreover, from gestational day 14 to PnD 14 SERT is momentarily expressed throughout the rat brain and the reason for this brief expression of SERT may be to maintain serotonin at a particular concentration in order to guide neurodevelopmental processes (Kepser & Homberg, 2015). Furthermore, rapid growth of serotonergic dendrites occurs after PnD 7 and by PnD 21 the adult pattern is already established (Murrin *et al.*, 2007). **Table 2-5** lists the neurodevelopmental processes that implicate serotonin.

Table 2-5: Neurodevelopmental processes that implicate serotonin (Kepser & Homberg, 2015).

Neurodevelopmental processes that implicate serotonin
• Enhancement of neurite outgrowth • Neurogenesis – in both a serotonin receptor- and region-specific way • Regulation of neuronal terminal development • Structural formation, as well as functioning of the somatosensory system and the hippocampus • Synaptogenesis

At birth, the serotonergic concentration in the central nervous system of the rat is generally low, peaks between PnD 21 and 30 (pre-pubertal period) and subsequently declines slightly to a concentration that resembles the serotonergic concentration in adult rats (Whitaker-Azmitia, 2001). These alterations in serotonergic concentration are comparable to the alterations seen in humans (Murrin *et al.*, 2007). Moreover, serotonin does not only exhibit the most rapid development compared to noradrenaline and dopamine (as mentioned earlier), but also the least dramatic (Murrin *et al.*, 2007).

With that said, serotonergic alterations during certain developmental stages may cause long-lasting changes in behaviour, as prenatal SSRI exposure in both rats and humans causes anxiety-like behaviour, as well as changes in social behaviour during adulthood (Olivier *et al.*, 2011). However, behavioural changes appear to be dependent on the specific SSRI administered and the route of SSRI administration (Kepser & Homberg, 2015). Moreover, alterations in the serotonergic concentration between birth and PnD 14 cause serious sensory impairments in addition to deficits in motor coordination, whereas serotonergic alterations between PnD 7 and 20 may lead to functional alterations and altered behaviour during later life (Andersen & Navalta, 2004). These findings are substantiated by other studies demonstrating that early-life elevations in serotonergic concentration result in an abnormal brain structure and function, motor coordination deficits, sensory impairments and behavioural abnormalities

(including depressive-like behaviour) during adulthood (Olivier *et al.*, 2011; Kepser & Homberg, 2015). Furthermore, clinical studies support the preclinical findings obtained from rodent models (Croen *et al.*, 2011; Kepser & Homberg, 2015).

Thus, the early maturation rate of the serotonergic system relative to the noradrenergic and dopaminergic systems suggests that antidepressants affecting the serotoninergic system may most likely have long-lasting (i.e. more advantageous or adverse) effects during early neurodevelopment compared to antidepressants affecting the noradrenergic and dopaminergic systems, especially considering the pivotal part serotonin plays in regulating neurodevelopment (Murrin *et al.*, 2007). However, very few studies have been conducted on the long-lasting effects of early-life (i.e. pre-pubertal and pubertal) pharmacological interventions (especially antidepressant treatment) and more research is warranted.

2.6.3.2.2 Noradrenergic development

Noradrenaline containing neurons are first detected in an 11 mm rat embryo (see **Figure 2-12**) and noradrenergic neurons differentiate between gestational day 10 and 13 (Murrin *et al.*, 2007). When a maximum number of noradrenergic neurons has been reached, enzymes responsible for noradrenaline and adrenaline synthesis are expressed (Murrin *et al.*, 2007). Moreover, during early postnatal development (first 21 days after birth) maturation of cortical noradrenergic neurons takes place and noradrenergic transporters (NERT) reach a concentration comparable to that observed in adult rats (Murrin *et al.*, 2007). During this period, noradrenergic innervation also increases to levels observed in adult rats, however noradrenergic innervations are only fixed in adult patterns between PnD 28 and 35 (Murrin *et al.*, 2007).

In the human brain, tyrosine hydroxylase is an enzyme required for noradrenaline synthesis and can be detected from week 4 of gestation, whereas noradrenalin is first detected between week 5 and 6 of gestation (Murrin *et al.*, 2007). Moreover, the different concentrations of noradrenaline at different stages of neurodevelopment in humans are comparable to that of the rat, as noradrenaline increases during the first trimester (particularly from the second month of gestation), followed by a reduction (30-40%) in noradrenergic concentrations from the sixth postnatal month to early childhood (Murrin *et al.*, 2007). Noradrenergic pathways develop and mature later than serotonergic pathways and this finding has been suggested to be the reason for the ineffectiveness of TCAs, MAOIs and SNRIs in the antidepressant treatment of children, whereas these antidepressants are effective in adults (Murrin *et al.*, 2007).

2.6.3.2.3 Dopaminergic development

Phenylalanine and tyrosine (i.e. amino acids) are required for the synthesis of dopamine in the presynaptic neuronal cytoplasm (Murrin *et al.*, 2007). Neuronal dopamine already occurs in the 9 mm rat embryo (see **Figure 2-12**) and dopaminergic neurons differentiate between gestation day 10 and 15 (Andersen, 2003). Moreover, between PnD 28 and 35 (pre-puberty) dopaminergic markers reach adult levels in the rat, including dopaminergic content, dopaminergic reuptake sites and tyrosine hydroxylase activity (Andersen, 2003). Furthermore, one study conducted on dopaminergic receptors during neurodevelopment in rats showed a consistent increase in dopaminergic receptors until a peak is reached between PnD 35 and 40 (puberty), followed by a reduction in dopaminergic receptors to levels seen in adult rats (Rho & Storey, 2001). However, in the human foetus, dopaminergic neurons are present from 6 to 8 weeks of gestation (Murrin *et al.*, 2007) and when compared to adults, the dopaminergic turnover during the perinatal period is relatively high (Herlenius & Lagercrantz, 2004).

Alterations in the development of dopaminergic pathways may lead to numerous long-lasting effects, for instance the adverse effects of the social isolation model (i.e. a developmental stressor) are ascribed to dopaminergic mechanisms, as constant dopaminergic alterations during a vulnerable stage of neurodevelopment may induce alterations in social behaviour, impairment of impulse control, deficits in cognitive control and impaired decision making during adulthood (Baarendse *et al.*, 2013). In addition, constant dopaminergic alterations during neurodevelopment may induce a loss of sensitivity to dopamine, disrupted neuroplasticity and alterations in dopaminergic neurotransmission, increasing an individual's vulnerability to develop MDD (Baarendse *et al.*, 2013).

2.6.3.2.4 Amino acid neurotransmitters

Although the role of monoaminergic neurotransmitter pathways in the aetiology of MDD is well described (see section 2.5.1.3), there is also evidence implicating amino acid neurotransmitters in the neuropathology of MDD, especially excitatory NMDA mediated glutamatergic and inhibitory gamma-aminobutyric acid (GABA) circuits (Choudary *et al.*, 2005; Harvey, 2006). Amino acid neurotransmitters can therefore have either an excitatory or inhibitory action on receptors (Choudary *et al.*, 2005; Harvey, 2006). Moreover, repeated excitatory postsynaptic potentials produce an action potential that enables neurons to transmit a signal from one to another, by releasing neurotransmitters from the presynaptic neuronal terminal into the synaptic cleft (Nieuwenhuys, 1994; Squire, 2003; Javitt, 2004). Also, glutamate is the primary excitatory neurotransmitter in the central nervous system and is used by nearly 60% of neurons in the brain (Nieuwenhuys, 1994; Squire, 2003; Javitt, 2004). Glutamate primarily acts on complex

postsynaptic NMDA receptors to bring about the down-stream release of NO and cyclic guanosine monophosphate (cGMP), following AMPA receptor provision of the initial depolarization required to unblock NMDA receptors and to enable calcium ion entry (Ca^{2+} influx) into the neuronal cell (Javitt, 2004). Furthermore, glutamate also acts on metabotropic receptors which support the regulation of both pre- and postsynaptic glutamatergic neurotransmission (Nieuwenhuys, 1994; Javitt, 2004).

Inhibitory ionotropic amino acid neurotransmitters include: GABA (i.e. a GABA receptor potentiator) and glycine (with an inhibitory action on NMDA receptors) (Leonard, 2003). Moreover, GABA inhibits neuronal signalling and the subsequent release of neurotransmitters into the synaptic cleft within the central nervous system (Leonard, 2003). However, biogenic amine neurotransmitters (i.e. serotonin, noradrenaline and dopamine) generally act on metabotropic receptors associated with intracellular second messenger systems (Minnaar, 2008), whereas ACh is a non-amino acid excitatory neurotransmitter and acts on ionotropic nicotinic and metabotropic muscarinic receptors (Leonard, 2003). Moreover, when high concentrations of neurotransmitters occur in the synaptic cleft (GABA, glutamate, serotonin, noradrenaline and dopamine), presynaptic inhibition of neurotransmitter release can also occur through the stimulation of auto-inhibitory α_2-adrenoceptors (Leonard, 2003).

2.6.3.2.5 Diverse neurotransmitters

Gaseous neurotransmitters (e.g. NO), unlike traditional neurotransmitters, are not kept in presynaptic vesicles and are not released from the presynaptic neuronal terminal through exocytosis (Vander *et al.*, 2001). Instead, NO is synthesized and released immediately upon up-stream glutamatergic signalling, after which it merely diffuses into adjacent neuronal terminals where it acts as a neuromodulator and neurotransmitter (Vander *et al.*, 2001). NO is described as a neuromodulator due to its ability to modify glutamatergic and several other signal transduction systems (Harvey, 1996; Prast & Philippu, 2001). In addition to NO's actions on neurotransmission, neuroplasticity and synaptic plasticity (Feil & Kleppisch, 2008; Kleppisch & Feil, 2009), preclinical studies have also implicated NO signalling in the regulation of several cognitive and emotional behaviours (Nelson *et al.*, 1995; Wiley *et al.*, 1995; Dzoljic *et al.*, 1997; Harkin *et al.*, 1999; Heiberg *et al.*, 2002).

Together with a low molecular weight, NO's hydrophobic properties enable it to easily diffuse over a distance of as much as 100-200 µm to adjacent neuronal cells, making NO a unique neurotransmitter (Meulemans, 1994; Schuman & Madison, 1994; Ledo *et al.*, 2004). Moreover, NO may be a mediator of neuronal plasticity (underlying brain development) and processes of information storage in the hippocampus, e.g. long-term potentiation (LTP) (Christopherson &

Bredt, 1997). Moreover, NO is described as a retrograde messenger, following the observation of LTP after the synthesis of NO at particular hippocampal sub-region postsynaptic synapses (O'dell *et al.*, 1991; Schuman & Madison, 1991; Arancio *et al.*, 1996; Ledo *et al.*, 2004) and the implication of NO in LTP is substantiated by other studies (Schuman & Madison, 1994; Boulton *et al.*, 1995; Christopherson & Bredt, 1997). Furthermore, LTP and other intracellular signalling pathways activated by glutamatergic NMDA receptor stimulation are in part responsible for regulating neurodevelopment (Dawson & Dawson, 1996; Dawson & Dawson, 1998) and also have other neuromodulatory actions, with NO playing an intricate part in these responses (Dawson & Dawson, 1996).

NO is an extremely unstable free radical, as it is oxidized rapidly to nitrite or nitrate when oxygenated haemoglobin, oxygen or superoxide are present (Helmke & Duncan, 2007) and therefore has a half-life of only a few seconds (Sun *et al.*, 2003; Jobgen *et al.*, 2007). In fact, NO's rapid reaction with molecular oxygen, superoxide and proteins that contain iron leads to the production of reactive intermediates, resulting in oxidative stress (Mayer *et al.*, 1995).

2.6.3.3 Theoretical framework for early-life drug-induced long-lasting effects

As mentioned in section 1.3, the primary objective of this study was to determine whether early-life (i.e. pre-pubertal and/or pubertal) exposure to the phosphodiesterase type 5 (PDE5) inhibitor, sildenafil, induces any later-in-life neurobehavioural and/or cognitive effects in the adult rats. Also, the current study aimed to ascertain the age at which sildenafil treatment (i.e. pre-pubertal and/or pubertal) induces the most robust bio-behavioural alterations in later life. Numerous neurodevelopmental hypotheses exist to both explain and predict such effects.

One hypothesis involves the process of synaptic overproduction and pruning during neurodevelopment, as previously mentioned. Numerous studies propose that the monoaminergic systems of mammals mainly demonstrate an overproduction of neurons, receptors and/or synapses during neurodevelopment, compared to levels observed in the adult animals. This elevated number of neurons, receptors and/or synapses later decrease to standard levels as seen in adult animals (Whitaker-Azmitia, 1991; Andersen *et al.*, 1997; Andersen *et al.*, 2000). The process of neuronal, receptor and synaptic decrease is known as pruning and the number of synapses lost during adolescence can be up to 40% (Huttenlocher, 1979a; Andersen *et al.*, 2000). Importantly, a period of vulnerability may be created by the overproduction and pruning process, in which the various brain regions are more susceptible to long-lasting effects resulting from drugs affecting these neurotransmission pathways (particularly in brain regions where greater overproduction and pruning occur) (Lidow & Song, 2001; Andersen, 2003).

It is important to mention that two additional hypotheses have been proposed related to the overproduction and pruning hypothesis. Firstly, the "Neural Darwinism" hypothesis proposes that the brain "selects" the synapses needed to be preserved into adulthood, which will eventually enable the brain to cope with the requirements of the environment (Piattelli-Palmarini, 1989; Edelman, 1993; Teicher, 2002). Secondly, the "Instructionist" hypothesis suggests that the brain is "instructed" to develop in a specific way by the environment, as established by the structural and/or functional needs of specific brain systems (Quartz & Sejnowski, 1997).

The synaptic development of the brain (i.e. sprouting, formation and growth) during early-life development is impacted by alterations in *serotonin* (Lauder & Krebs, 1978; Kuppermann & Kasamatsu, 1984; Whitaker-Azmitia & Azmitia, 1986), *noradrenaline* (Feeney & Westerberg, 1990; Kline *et al.*, 1994) *and dopamine* (Kalsbeek et al., 1988; Lankford et al., 1988; Gelbard et al., 1990; Todd, 1992) concentrations. Thus, early-life exposure to drugs that influence these neurotransmitter concentrations may cause effects that only manifest in later life (Andersen & Navalta, 2004).

2.7 Treatment

MDD is not only associated with dire psycho- and bio-pathology and often unsatisfactory pharmacotherapeutic response, but also with poor health indicators related to life-style, including smoking, physical inactivity and high caloric consumption (Bonnet *et al.*, 2005; Abildgaard *et al.*, 2011). Consequently, there is an increased drive for research into novel antidepressants and augmentation strategies, not only for the treatment of the disorder, but also to reduce the risk of developing MDD (Gersing *et al.*, 2014).

There are currently several treatment strategies for the treatment of MDD, including psychotherapeutic, pharmacological (i.e. antidepressants), lifestyle and adjuvant or alternative approaches, each with different degrees of effectiveness and risks (Marais *et al.*, 2009; Willner *et al.*, 2013). In an attempt to improve the efficacy of antidepressant therapies, several augmentation strategies have been developed. Augmentation strategies include both pharmacological and non-pharmacological treatment options (Hoagwood *et al.*, 2001). Pharmacological augmentation strategies include the addition of another first-line antidepressant or a second-generation antipsychotic to initial antidepressant treatment, whereas non-pharmacological augmentation strategies include psychotherapy, electroconvulsive therapy (ECT), sleep deprivation, deep brain stimulation and lifestyle modifications (Hoagwood *et al.*, 2001).

Pharmacological treatment is frequently used as first-line therapy to treat overt MDD, whereas non-pharmacological interventions (for example psychotherapy, life-style adjustments and support groups) are either used as an augmentation strategy or in monotherapy of mild MDD (Marais *et al.*, 2009; Willner *et al.*, 2013). Psychotherapy includes psychosocial interventions (e.g. cognitive behavioural therapy, behavioural activation treatment, nondirective supportive treatment, problem-solving therapy, psychodynamic treatment, interpersonal psychotherapy and social skills training) (Cuijpers *et al.*, 2008) and relaxation techniques, whereas lifestyle adjustments frequently include biophysical interventions, such as dietary optimization and exercise (Willner *et al.*, 2013).

Although MDD can be effectively treated in most individuals with either pharmacological therapies and/or some form of evidence-based psychotherapy (American Psychiatric Association, 2013), up to 20% of individuals that suffer from MDD fail to respond to standard antidepressant treatment options (Fava, 2003), known as treatment resistance. However, there are some treatment strategies that have been shown to be effective in treating treatment-resistant depression, viz. atypical antipsychotics (e.g. risperidone, ziprasidone, olanzapine and quetiapine) (Kennedy & Lam, 2003), deep brain stimulation (Mayberg *et al.*, 2005), ECT (Mayberg *et al.*, 2005; Little, 2009) and vagus nerve stimulation (Little, 2009), all associated with significant risk, side effects and/or different levels of invasiveness.

Pharmacotherapy remains the first-line approach in moderate to severe MDD (Willner *et al.*, 2013) and the treatment of paediatric MDD is especially complicated due to the vulnerability of juveniles, uncertainty about the safety and efficacy of antidepressant treatment and limited approved pharmacological treatment options (Marais *et al.*, 2009). There is great need to optimise antidepressant treatment strategies in childhood and adolescence to reduce the risk of relapse (Marais *et al.*, 2009).

The pharmacotherapy with regards to MDD in general, and in particular the SSRIs in the treatment of paediatric MDD, will be discussed in section 2.7.1 below.

2.7.1 Pharmacotherapy

All antidepressants that are currently commercially available are classified according to the neurobiological target applicable, in nearly all instances according to the effect these antidepressants have on the monoaminergic system (Willner *et al.*, 2013). The various classes of antidepressants, with drug examples, are listed in **Table 2-6** below:

Table 2-6: Classes of antidepressant drugs used in the treatment of MDD, with drug examples (Willner *et al.*, 2013).

Class	Drug examples
1. **Monoamine oxidase inhibitors (MAOI)**	Tranylcypromine; phenelzine; selegiline; rasagiline, moclobemide and brofaromine
2. **Tricyclic antidepressants (TCA)**	Amitriptyline; imipramine
3. **Selective serotonin reuptake inhibitors (SSRI)****	Fluoxetine*; paroxetine, fluvoxamine; sertraline; citalopram; escitalopram*
4. **Serotonin and noradrenalin reuptake inhibitors (SNRI)****	Venlafaxine; duloxetine
5. **Noradrenalin reuptake inhibitors (NARI)**	Atomoxetine; reboxetine
6. **Dopamine reuptake Inhibitors**	Bupropion
7. **Serotonin receptor type 2 ($5HT_2$) antagonists**	Trazodone; nefazodone
8. **Tetracyclics and unicyclics**	Mirtazapine; amoxapine
9. **Atypical drugs**	Agomelatine; tianeptine, vortioxetine
10. **Herbals**	St. John's wort (*Hypericum perforatum*)

* Antidepressant drugs approved by the FDA for use in children and adolescents (Oberlander & Miller, 2011; Soutullo & Figueroa-Quintana, 2013)

** Classes of antidepressant drugs that form part of the first-line pharmacotherapy for MDD (NIMH, 2011)

Figure 2-14 below illustrates central noradrenergic (top) and serotonergic (bottom) synapses with monoaminergic receptors and neurotransmitters. Each of the aforementioned five classes of antidepressants are discussed in more detail below, with reference to each class's mechanism of antidepressant action (see **Figure 2-14**).

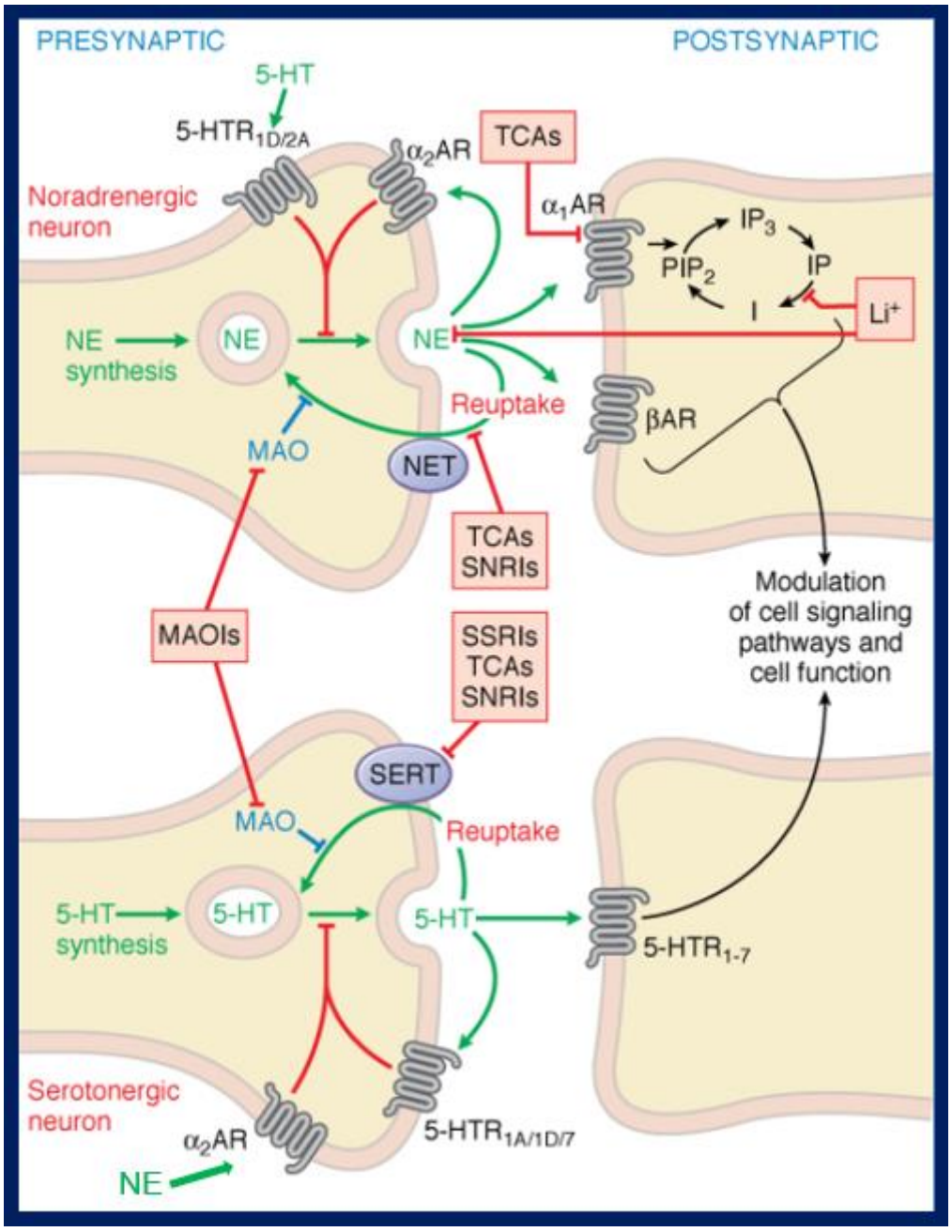

Figure 2-14: Sites of antidepressant action (Brunton *et al.*, 2011). With abbreviations: 5-HT = serotonin, NE = noradrenaline, SSRI = selective serotonin reuptake inhibitor, SNRI = serotonin-noradrenalin reuptake inhibitor, TCA = tricyclic antidepressant, MAO = monoamine oxidase, MAOI = monoamine oxidase inhibitor, SERT = serotonin reuptake transporter, NET = noradrenaline reuptake transporter, 5-HTR = serotonin receptor, αAR = alpha-adrenergic receptor and βAR = beta-adrenergic receptor.

Despite the wide variety of antidepressants that are available for the treatment of MDD in adults, only two of these drugs are suitable for the treatment of paediatric MDD, as previously mentioned. With that said, the FDA has only approved fluoxetine to treat MDD in children (7-12 years) and fluoxetine and escitalopram to treat adolescent MDD (12-18 years) (Oberlander & Miller, 2011; Soutullo & Figueroa-Quintana, 2013). A limited amount of data is available on the usage of antidepressants in children and adolescents, due to historical approaches, suggesting

children primarily being treated as “little adults” (Cheung *et al.*, 2005), as well as obvious ethical implications and considerations for clinical trials, resulting in a lack of suitable treatment options for juvenile MDD. In addition, the small amount of research conducted on the use of SSRIs to treat MDD in children has many shortcomings, as nearly all clinical trials testing the efficacy of SSRIs in treating childhood MDD are of short duration, have a small number of participants and/or are industry funded (i.e. conflict of interest), resulting in a limited ability to detect and/or report major adverse events (Kastelic *et al.*, 2000).

The FDA now requires paediatric research data for all new compounds intended to treat MDD in children before approval of such compounds and encourages paediatric research on existing compounds (Cheung *et al.*, 2005; Libby *et al.*, 2007). Consequently, the amount of data available for a number of existing antidepressants have increased. However, controversy still exists about the safety and the efficacy of antidepressant-use in juvenile patients. The biggest concern is an increase in suicidal ideation seen in children and adolescents (under the age of 18 years) during the first few weeks of antidepressant treatment (Libby *et al.*, 2007; Klomp *et al.*, 2014).

SSRIs are currently the most prescribed antidepressants, not only in children, but in all age groups, due to the relative safety and superior side-effect profile of the SSRIs in comparison with the older antidepressants (i.e. MAOIs and TCAs) (Klomp *et al.*, 2014). Important to note, SSRIs are the only antidepressant drugs that prove effective in children according to meta-analyses, as well as randomised control trials (Thapar *et al.*, 2012). The superior effectiveness of the SSRIs over the TCAs in children has been ascribed to the different maturation rates of the monoaminergic pathways (i.e. serotonergic and noradrenergic) in the developing brain and will be discussed in more detail in this dissertation (see section 2.6.3). It is important to note that the first-line pharmacological therapies (i.e. SSRIs and SNRIs) do not show greater efficacy in adults when compared to older classes of antidepressants, but have a better side-effect profile (Millan, 2006; NIMH, 2011).

As alluded to earlier, the wide range of different antidepressants currently commercially available does not meet all of the clinical needs. Shortcomings include a delayed onset of antidepressant action, ineffectiveness in both refractory patients and individuals suffering from treatment-resistant depression, a limited reduction in *cognitive deficits* caused by MDD and a troublesome side-effect profile (Pacher & Kecskemeti, 2004; Rosenzweig-Lipson *et al.*, 2007). Moreover, a transient condition known as antidepressant discontinuation syndrome occurs following abrupt withdrawal or a reduction in antidepressant dose and causes troublesome symptoms, in particular flu-like symptoms (Warner *et al.*, 2006). In addition, premature

withdrawal may not only result in relapse, but may also increase the risk of developing treatment resistant MDD (Adli *et al.*, 2003; Mann, 2005; Adli *et al.*, 2006).

There are five major classes of antidepressants (i.e. SSRIs, SNRIs, MAOIs, TCAs and atypical antidepressants) and each class differs from the other classes with regards to clinical efficacy and side-effect profile. The drive behind the search for novel antidepressants is the need for antidepressants with a faster onset of action, as well as the need for antidepressants that exhibit greater tolerability and are proven effective in treatment-resistant MDD.

2.7.1.1 Selective serotonin reuptake inhibitors

In the late 1960s it was suggested that the central nervous system neurotransmitter serotonin may be a suitable target for antidepressant therapy (Carlsson *et al.*, 1968; Lapin & Oxenkrug, 1969). This revelation was instrumental in the discovery of a number of SSRIs during the early 1980s, most notably fluoxetine. Finally, nearly 20 years after its discovery, fluoxetine became commercially available under the trade name Prozac® (Stokes & Holtz, 1997). A number of SSRIs soon followed and are commonly used as antidepressants today, viz. paroxetine, citalopram and sertraline (Fuller, 1995; Ferguson, 2001; Kasper *et al.*, 2009).

SSRIs, primarily, treat MDD by inhibiting the reuptake of serotonin, through inhibition of serotonin transporters, from the synaptic cleft into the pre-synaptic terminal, resulting in an increase in the serotonin concentration within the synaptic cleft (see **Figure 2-14**), regulation of postsynaptic G protein-coupled receptors, which couple to a variety of second messenger systems, over-stimulation and subsequent desensitisation of pre- and post-synaptic serotonin receptor subtypes. This leads to a consequential modulation of serotonergic neurotransmission, associated with antidepressive effects (Brunello *et al.*, 1994; Leonard, 1995; Goodwin, 1996; Krishnan & Nestler, 2008). Serotonin transporters represent the main target for the SSRIs and have a critical function in regulating mood (Ansorge *et al.*, 2004). In fact, a reduction in the expression of serotonin transporters has been linked to neuroticism, anxiety-like behaviour and depressive-like symptoms (Ansorge *et al.*, 2004).

The stimulation of $5HT_{1A}$ auto-receptors and $5HT_{1B}$ receptors have been reported to be pivotal targets for the antidepressive-like effects observed after chronic SSRI exposure (Artigas *et al.*, 1996; Blier, 2003). The aforementioned receptors suppress serotonin synthesis, but persistent stimulation of these receptors results in continuous down-regulation and desensitization, eventually giving rise to an increased synthesis and release of serotonin (Blier & Chaput, 1987; Chaput *et al.*, 1991). The $5HT_3$ receptor subtype has been associated with many of the side effects observed with SSRI treatment, including gastrointestinal and sexual adverse effects,

whereas agitation and restlessness may be related to the stimulation of the $5HT_{2C}$ receptor subtype (Baldessarini, 1996).

Moreover, fluoxetine decreases dopamine transmission through the down-regulation of $5HT_{2B/2C}$ receptor subtypes (Dailly *et al.*, 2004) and dopamine transmission is therefore inhibited prior to the development of tolerance to the effects of serotonin on the $5HT_2$ receptor (Dailly *et al.*, 2004). This indirect effect that SSRIs have on dopaminergic neurotransmission may in part explain the delay in onset of antidepressant action seen with SSRI treatment (Dailly *et al.*, 2004; Karanges & McGregor, 2011). Dopamine plays a significant role in the *anhedonic* characteristics related to MDD and an elevation in dopaminergic transporter expression has been demonstrated following chronic SSRI treatment (Rominger *et al.*, 2015). Moreover, SSRIs have an extremely low affinity for dopaminergic receptors and transporters, however SSRIs have acute and chronic effects on dopaminergic function. This is ascribed to serotonergic stimulation (in several brain regions) resulting in the potent release of dopamine, which has been closely associated with an antidepressant effect (Renard *et al.*, 2001).

In addition, SSRIs have also been demonstrated to display anti-inflammatory properties (Walker, 2013), decreasing the inflammatory cytokines interleukin (IL)-1 and IL-6 (Hannestad *et al.*, 2011), and increasing the anti-inflammatory cytokine IL-10 (Janssen *et al.*, 2010). SSRIs have further been shown to have anti-oxidative properties, decreasing malondialdehyde (MDA) and superoxide dismutase (SOD) in human patients following fluoxetine treatment (Bilici *et al.*, 2001; Khanzode *et al.*, 2003). Serotonin can activate both downstream signalling pathways and transcription factors that in turn affect the expression of proteins related to the regulation of neural plasticity, stress resistance and cell survival during adulthood in humans and rodents (Jin *et al.*, 2009; Marais *et al.*, 2009; Harmer & Cowen, 2013).

2.7.1.1.1 SSRIs in the treatment of childhood depression

As alluded to earlier, SSRIs (in the case of children and adolescents fluoxetine and escitalopram) inhibit the serotonin reuptake transporter protein (Willner *et al.*, 2013) and therefore inhibit the synaptic reuptake of serotonin from the synaptic cleft into the presynaptic neuron, resulting in an increased concentration of serotonin in the synaptic cleft (Baldessarini, 2006). Thus, serotonin is not transported back to where the storage vesicles are located within the presynaptic neuron (Walker, 2013) and subsequent stimulation and desensitisation of pre- and post-synaptic monoaminergic receptor subtypes occur, with consequential modulation of monoaminergic neurotransmission, associated with antidepressive effects (Brunello *et al.*, 1994; Leonard, 1995; Goodwin, 1996; Harvey, 1997; Krishnan & Nestler, 2008) and this can be seen illustrated in **Figure 2-15** below.

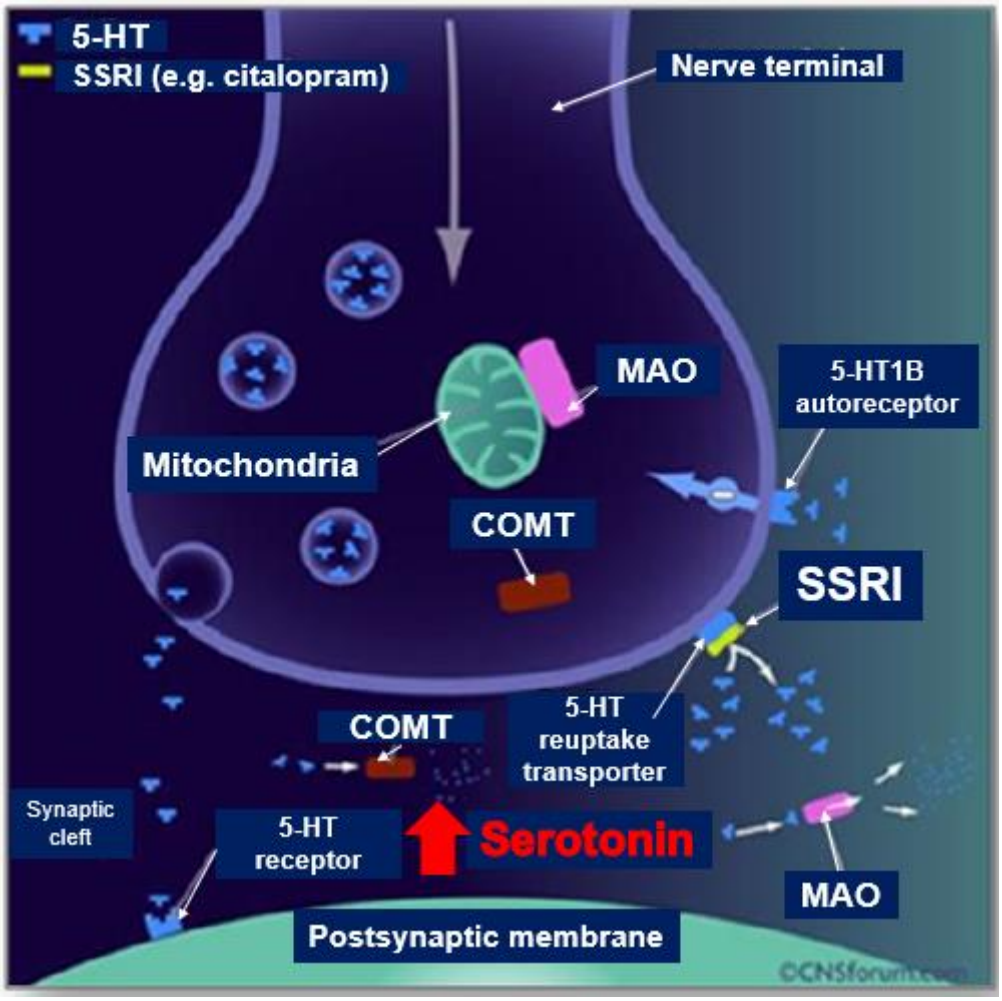

Figure 2-15: SSRIs inhibit serotonin reuptake, causing an increase in the concentration of serotonin within the synaptic cleft. Adapted from (Rang *et al.*, 1995; Duman & Voleti, 2012). With abbreviations: 5-HT = serotonin, SSRI = selective serotonin reuptake inhibitor, MAO = monoamine oxidase and COMT = catechol-O-methyltransferase.

Despite the immediate SSRI-induced elevation in serotonin concentrations within the synaptic cleft, symptoms of MDD are only alleviated after 2-4 weeks of antidepressant treatment, whereas several months are required for complete remission (Stahl, 1998; Berton & Nestler, 2006a; Iñiguez *et al.*, 2010; Kovačević *et al.*, 2010; Harmer & Cowen, 2013). Therefore, long-term treatment of months, or in some cases years, is required with SSRI therapy and inevitably juvenile antidepressant treatment may have a profound impact on neurodevelopment, influencing neurobiological functioning later in life (Iñiguez *et al.*, 2010). In fact, a twelve-month treatment period is recommended for children and adolescents suffering from MDD, correlating with the recommendation for adults (Pine, 2002).

Lasting effects of juvenile fluoxetine administration on neuroanatomy have been reported (Andersen & Navalta, 2004). Worth mentioning is that these lasting effects on neuroanatomy are consistent with the ***"equal, but opposite"* hypothesis**. This hypothesis is based on

observations in adult animals where chronic drug exposure leads to an accommodation to the effects of the drug, culminating from an array of compensatory reactions, whereas chronic drug exposure in juvenile animals results in assimilation, by incorporating drug-induced alterations in the form of permanent developmental changes in the system (Andersen & Navalta, 2004). Therefore, early-life exposure to SSRIs may result in lasting alterations in the serotonin system (that can be observed during adulthood), however the lasting effects of SSRI treatment in juveniles have produced inconsistent results that are largely dependent on the brain region investigated (Andersen & Navalta, 2004).

Serotonin-related behavioural alterations are also seen in adult rats following chronic pubertal SSRI treatment, including altered body weight, decreased sexual functioning and increased anxiety-like behaviour (De Jong *et al.*, 2006). In addition, affected responsiveness to rewarding and/or aversive stimuli during adulthood has also been documented after chronic SSRI treatment in early-life (Iñiguez *et al.*, 2010). These intricate functional outputs are regulated by a number of factors, including the environment in which they are encountered, the brain circuitry involved and the emotional valence of the stimulus (Iñiguez *et al.*, 2010). Moreover, findings from animal studies show that fluoxetine-induced anxiety-like behaviour can be attenuated by re-exposure to fluoxetine (Iñiguez *et al.*, 2010).

Paediatricians, child psychiatrists and general practitioners still face the challenging task of prescribing antidepressants to young children without understanding the long-lasting effects of administering antidepressants during certain periods of neurodevelopment (Marais *et al.*, 2009). Also, there is no single antidepressant that is effective for all and this further highlights the need for alternative treatment options and/or augmentation strategies to treat juvenile MDD, with improved safety and efficacy profiles (Marais *et al.*, 2009). Moreover, antidepressant treatment strategies need to be optimised in children to reduce the risk of relapse later in life.

Furthermore, it has been suggested that SSRI monotherapy be used as first-line treatment in children suffering from MDD, and in the event that the first SSRI is unsuccessful, it be substituted with an alternative SSRI (Hughes *et al.*, 1999). In addition, it has been proposed that children and adolescents with SSRI-resistant depression be treated with the SNRI, venlafaxine, as it has a comparable antidepressant efficacy to that observed in SSRI responsive juveniles (Brent *et al.*, 2008). However, TCA treatment in children and adolescents has no superiority to placebo-treated groups (Kutcher *et al.*, 1994; Kye *et al.*, 1996; Keller *et al.*, 2001) and therefore has no value in the treatment of juvenile MDD.

In conclusion, SSRIs, more specifically fluoxetine and escitalopram, are the preferred antidepressant drugs in the treatment of paediatric MDD and these drugs must be administered

for a prolonged period of time in order to improve the symptoms of MDD, and this may be associated with long-lasting neurodevelopmental effects.

2.7.1.2 The serotonin-noradrenaline reuptake inhibitors

The SNRIs, including venlafaxine, duloxetine and milnacipran, were developed to inhibit the presynaptic reuptake of both serotonin and noradrenaline, with the hope that they will display enhanced efficacy relative to SSRIs and fewer side effects than the older TCAs and MAOIs (Bauer *et al.*, 2009). Of these, venlafaxine was the first to be introduced and has been suggested to be more effective than the SSRIs in some patients with treatment resistance (Bauer *et al.*, 2009).

Moreover, the monoaminergic hypothesis for the neurobiological basis of MDD (see section 2.5.1.3) implicates both serotonin and noradrenaline in the pathophysiology of MDD (Montgomery, 1997; Bylund & Reed, 2007). In this regard, the SNRIs inhibit both serotonin and noradrenaline reuptake (see **Figure 2-14**). Since respective SNRIs display different selectivity for serotonergic and noradrenergic reuptake transporter proteins, dual inhibition is dose-dependent, and sometimes requires higher doses to display optimal antidepressant effects (Gur *et al.*, 1999; Entsuah *et al.*, 2001; De Oliveira *et al.*, 2004; Merck, 2006).

Some studies propose that the SNRIs, especially venlafaxine, may have a faster onset of antidepressant action compared to the other classes of antidepressants (Feighner, 1994; Montgomery, 1995). A number of studies have shown an initial clinical response to venlafaxine as early as fourteen days after starting with treatment, compared to twenty one days observed with the SSRIs (Rudolph et al., 1991; Clerc et al., 1994; Guelfi et al., 1995; Benkert et al., 1996; Benkert et al., 1997). Venlafaxine is also a popular 'off-label' antidepressant in treating juvenile MDD (Volkers *et al.*, 2007; Zito *et al.*, 2008; Lee *et al.*, 2012) with potent anxiolytic properties in juvenile patients (March et al., 2007; Rynn et al., 2007).

In addition to antidepressant effects, venlafaxine has analgesic effects (Enggaard *et al.*, 2001) whereas duloxetine is also used in the treatment of urinary incontinence (Brunton *et al.*, 2010a).

2.7.1.3 The monoamine oxidase inhibitors

Monoamine oxidase (MAO) is an enzyme that occurs in the outer mitochondrial membrane of both neuronal and non-neuronal cells (see **Figure 2-14**). MAO has two isoforms that occur in the human body, namely MAO-A and MAO-B (Schildkraut, 1965; Ruhé *et al.*, 2007; Finberg & Gillman, 2011). Both isoenzymes (i.e. MAO-A and MAO-B) are present in the central nervous system, as well as several peripheral organs. In this regard, MAO-A is found peripherally in the

liver, heart and pancreas, whereas MAO-B is present in the liver and pancreas. With regards to the central nervous system, MAO-A is found in both noradrenergic and dopaminergic neurons, whereas MAO-B is predominantly present in serotonergic neurons. With that said, MAO-A has a greater peripheral distribution, whereas MAO-B is found more abundantly in the central nervous system (Krishnan, 2017)

Both of these isoenzymes are responsible for degrading biogenic amines (i.e. noradrenaline, serotonin and dopamine) in the synaptic cleft, resulting in reduced concentrations of these biogenic amines within the synaptic cleft and according to the monoaminergic hypothesis (see section 2.5.1.3), this reduction leads to the development of MDD (Schildkraut, 1965; Ruhé *et al.*, 2007; Finberg & Gillman, 2011). The MAOIs inhibit these particular enzymes (i.e. MAO-A and MAO-B) in the pre-synaptic neurons, leading to increased levels of monoamines within the synaptic cleft (Schildkraut, 1995; Katzung, 2007b), with subsequent stimulation and desensitisation of pre- and post-synaptic monoaminergic receptor subtypes and consequential modulation of monoaminergic neurotransmission, associated with antidepressant-like effects (Krishnan & Nestler, 2008; Steyn, 2011). In fact, mood-elevating effects have been demonstrated following MAOI exposure as early as the 1950s (Crane, 1957; Kline, 1958).

MAO-A mainly catabolises noradrenaline and serotonin and MAO-A selective inhibitors (i.e. moclobemide and brofaromine) are primarily used as antidepressants (Lotufo-Neto *et al.*, 1999), whereas MAO-B selective inhibitors (i.e. selegeline and rasagiline) (Knoll & Magyar, 1972; Knoll *et al.*, 1978) are used to treat Parkinsonism, since MAO-B metabolises dopamine preferentially (Brunton *et al.*, 2010b). Moreover, selegeline has demonstrated potential as an antidepressant at higher dosages in Parkinsonism patients with co-morbid MDD (Katzung, 2007b).

However, the older MAOIs (tranylcypromine and phenylzine) non-selectively inhibit both MAO-A and MAO-B (Finberg & Gillman, 2011). When taken with dietary tyramine (a monoaminergic precursor), these drugs induce a hypertensive crisis, as there is an acute increase in monoaminergic concentrations throughout the body, without the ability of MAO to degenerate these high levels of monoamines. The term “cheese effect” is commonly used to describe this hypertensive crisis (Finberg & Gillman, 2011).

It can therefore be concluded that the MAOIs enhance monoaminergic neurotransmission and thereby decrease depressive-like symptoms, in accordance with the monoaminergic hypothesis.

2.7.1.4 The tricyclic antidepressants

The TCAs have a distinctive three-ring nucleus and have been used in antidepressant therapy for more than thirty years (Katzung, 2007b; Reay *et al.*, 2010). The TCAs are chemically

comparable to the phenothiazines and therefore have similar side effect profiles (Leonard, 1997). The antidepressant activity of these drugs was first demonstrated in schizophrenic patients, where beneficial effects on their depressive-like symptoms were observed following TCA treatment (Hollister, 1981).

The TCAs are divided into two subclasses, namely secondary and tertiary amines, which vary in their spectrum of selectivity for reuptake transporters and activity on a wide range of receptors. Moreover, the tertiary amines inhibit both noradrenergic and serotonergic reuptake (by inhibiting both noradrenergic and serotonergic reuptake transporters, see **Figure 2-14**), whereas the secondary amines preferably inhibit noradrenergic reuptake (by inhibiting noradrenergic reuptake transporters, see **Figure 2-14**) (Merck, 2006; Brunton *et al.*, 2010a). In addition, the TCAs also have affinities for histaminergic (H_1), alpha-adrenergic (α) and muscarinic (M) receptors (TCAs are multipotent) and the inhibition of these receptors contributes to an increased side effect profile for the TCAs (Wijeratne & Sachdev, 2008).

Tertiary amines (e.g. amitriptyline, clomipramine and imipramine) are metabolised to secondary amines (e.g. desipramine, nortriptyline and maprotiline) by the liver, which are pharmacologically active and give rise to both therapeutic and adverse effects (Reay *et al.*, 2010). The secondary amines, however, are metabolised to pharmacologically inactive compounds (Reay *et al.*, 2010). In conclusion, TCAs became an indispensable part of antidepressant therapy, but a very unfavourable side effect profile warranted the development of the SSRIs. Nevertheless, the TCAs are still used as an alternative to the SSRIs in the treatment of MDD today.

2.7.1.5 The atypical antidepressants

The atypical antidepressants can be described as drugs with both unrelated chemical structures and mechanisms of antidepressant action (Kent, 2000) and that differ from those discussed above. The atypical antidepressants were developed due to the need for antidepressants with a superior efficacy, less side effects and an earlier onset of antidepressant action (Kent, 2000). Moreover, due to inter-individual differences, the atypical antidepressants may in some cases be useful in patients that do not respond to other antidepressants, however they all affect monoaminergic neurotransmission in some way or another, and have not been shown to be superior to other antidepressants in general (Kent, 2000).

2.7.2 The search for novel antidepressants

Despite the multiple pharmacological treatment options available for the treatment of MDD (see section 2.7.1), remission following first-line antidepressant therapy is only accomplished in 30-

50% of patients (Solé *et al.*, 2015), whereas a therapeutic response is only reached in around 60-70% of patients (Ménard *et al.*, 2016). This can be ascribed to behaviour-alone diagnostic methods and/or the lack in specificity of current antidepressant therapies (Nestler *et al.*, 2002; Ménard *et al.*, 2016). Moreover, many of the patients that respond favourably to antidepressant therapy still experience subsyndromal symptomatic depression (Solé *et al.*, 2015). In addition to several safety and pharmacokinetic concerns, limitations of current antidepressants also include a delayed onset of antidepressant action, treatment resistance and toxicity (Nestler *et al.*, 2002; Drevets *et al.*, 2008; Machado-Vieira *et al.*, 2009; Banasr *et al.*, 2011; O'Donnell & Shelton, 2011; Sadaghiani *et al.*, 2011; Réus *et al.*, 2015; Solé *et al.*, 2015). Not surprisingly, 30% of patients do not respond to conventional antidepressant therapies (Solé *et al.*, 2015).

This emphasises the necessity for investigations into novel targets/mechanisms involved in the pathologies underlying MDD, as well as novel augmentative strategies. In this regard, some novel treatment and augmentative strategies are listed in **Table 2-7** below. See O'Leary and colleagues (2015) for a complete review of novel antidepressant targets/mechanisms, as well as novel augmentative strategies implicated in MDD (O'Leary *et al.*, 2015).

Table 2-7: Novel treatment and augmentative strategies, with examples, for MDD. Adapted from (Quirk & Nisenbaum, 2002; Kramer *et al.*, 2004; Bacchi *et al.*, 2006; Hodgson *et al.*, 2007; Brink *et al.*, 2008; Koo & Duman, 2008; Covington *et al.*, 2009; Skuza & Rogóż, 2009; Liebenberg *et al.*, 2010a; O'Leary & Castrén, 2010; Bravo *et al.*, 2011; Li *et al.*, 2011; Maes, 2011a; Mnie-Filali *et al.*, 2011; Owenby *et al.*, 2011; Felice *et al.*, 2012; Jutkiewicz & Roques, 2012; Tran *et al.*, 2012; Chang *et al.*, 2013; Drevets *et al.*, 2013; Mørk *et al.*, 2013; O'brien *et al.*, 2013; Ota & Duman, 2013; Pilc *et al.*, 2013; Risinger *et al.*, 2014; Walker *et al.*, 2015).

Novel treatment and augmentative strategies for MDD	Examples
Novel targeting strategies	• Corticotropin-releasing factor-1 receptor antagonism • Galanin (serotonin and noradrenaline neurotransmission modulator) agonism or antagonism • Gamma-aminobutyric acid-B ($GABA_B$) receptor antagonism • Ghrelin modulation • G-protein-coupled vasopressin-1_A receptor antagonism • Immunomodulation (e.g. cyclooxygenase inhibitors) • Melanin-concentrating hormone-1 receptor antagonism • Neurokinin-2 receptor antagonism • Neuropeptides, with regard to: - HPA axis regulation - monoaminergic neurotransmission modulation • Neuropeptide Y_2 antagonism • **NO-cGMP pathway** (see section 2.8 below) - **PDE5-selective inhibitors (e.g. sildenafil)** • Opioid receptor antagonism • Orexin-2 receptor antagonism • Oxytocin agonism • Psychobiotics/probiotics • Purine receptor antagonism • Sigma-1 receptor agonism
Novel multimodal targeting strategies	• Novel SSRIs: - Vilazadone (multipotent SSRI and partial $5HT_{1A}$ receptor agonist - Vortioxetine (partial $5HT_{1B}$ receptor agonist, $5HT_{1A}$ receptor agonist and $5HT_{3+7}$ receptor antagonist - *with procognitive effects*)

Novel treatment and augmentative strategies for MDD	Examples
	• Reversible MAO-A inhibitors: - CX157 - meclobemide • Triple reuptake inhibitors (serotonin, noradrenaline and dopamine reuptake inhibitors): - amitifadine - BMS-820836
Novel augmentation strategies	• Atypical antipsychotics in combination with SSRIs • Exercise • Lithium or triiodothyroxine in combination with TCAs • Permeability glycoprotein inhibition in combination with one of the following antidepressants: - desimipramine - escitalopram - imipramine - nortriptyline • Supplementation with omega-3 polyunsaturated fatty acids
Novel therapeutic onset rate enhancing strategies	• ACh action modulation through: - muscarinic receptor modulation - nicotinic receptor modulation • Chronotherapeutics/modulation of circadian rhythm • Glutamate neurotransmission modulation through: - glial-cell glutamatergic transporter augmentation - metabotropic glutamatergic receptor modulation - NMDA and AMPA receptor modulation
Underlying molecular and pathophysiological mechanisms of MDD as novel targeting strategies	• Epigenetic modifications • FK506 binding protein-5 (protein coding gene for MDD) modulation • Glycogen synthase kinase-3 inhibition • Neurotrophic factors (e.g. BDNF) and associated signalling pathways • Potassium channel subfamily K member 2 inhibition

2.8 The Glu-NO-cGMP-PK-G pathway and the pathophysiology of MDD

Since the current study investigates central effects of the PDE5 inhibitor sildenafil, which modulates the Glu-NO-cGMP-PK-G pathway, this pathway will be discussed in more detail. Glutamatergic neurotransmission is significantly implicated in MDD, as previously discussed (see section 2.5.1.5) and the modulation of the Glu-NO-cGMP-PK-G pathway shows promise as a mood regulating strategy (Zarate *et al.*, 2002; Zarate *et al.*, 2003; Sanacora *et al.*, 2008). Despite most research having been conducted on the role that glutamate and its receptors play in the pathophysiology of MDD, studies have also researched the more downstream mechanisms of glutamatergic neurotransmission that play a role in MDD (Zarate *et al.*, 2002; Zarate *et al.*, 2003; Sanacora *et al.*, 2008). In this regard, the role that glutamate, its receptors and the downstream mechanisms involved in glutamatergic neurotransmission play in the pathophysiology of MDD will be discussed in great detail below. **Figure 2-16** below illustrates the Glu-NO-cGMP-PK-G signalling pathway.

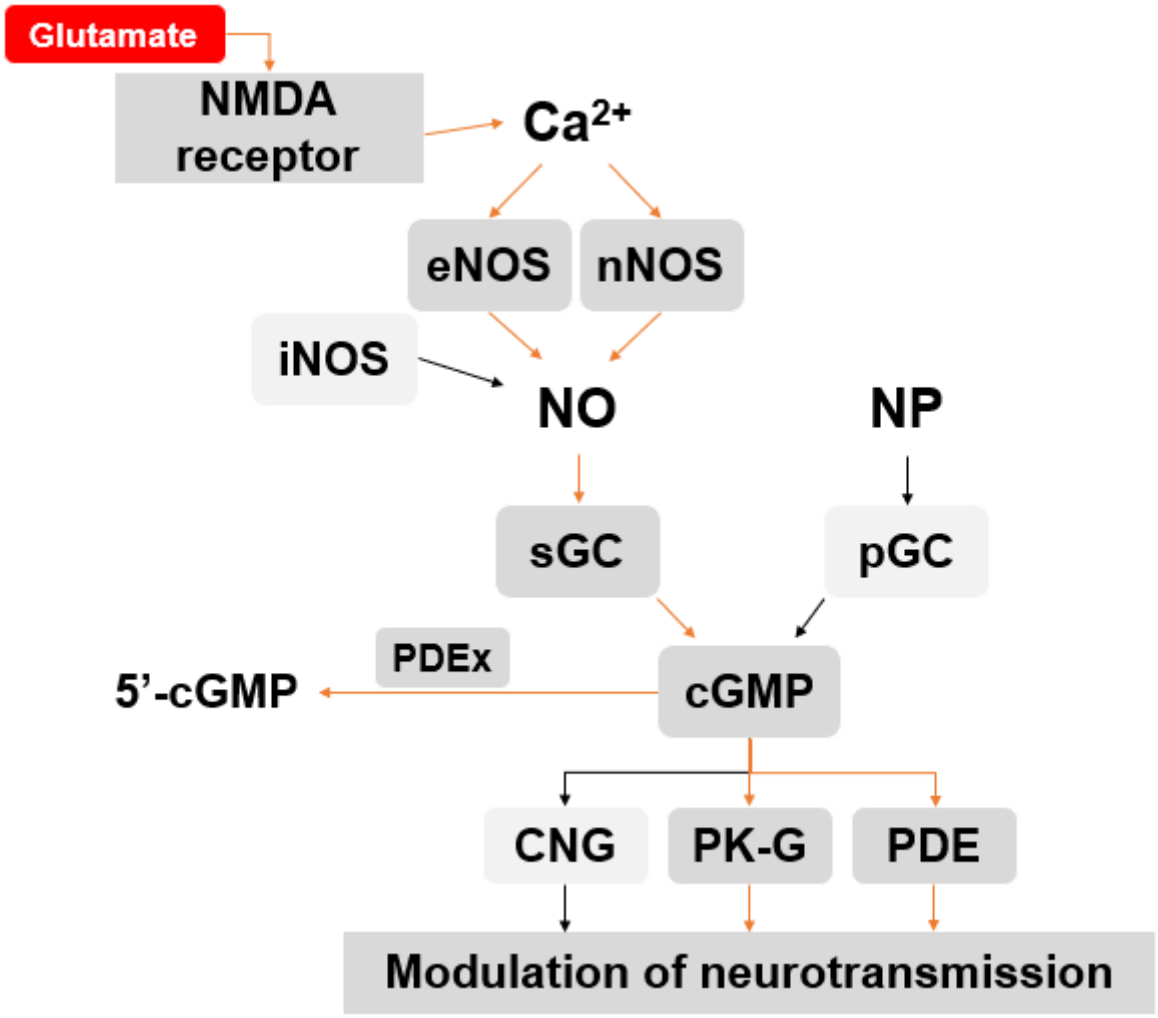

Figure 2-16: Illustration of the Glu-NO-cGMP-PK-G signalling pathway (Feil & Kleppisch, 2008). With abbreviations: NMDA = N-methyl-D-aspartate, NO = nitric oxide, iNOS = inducible nitric oxide synthase, eNOS = endothelial nitric oxide synthase, nNOS = neuronal nitric oxide synthase, sGC = soluble guanylyl cyclase, NP = natriuretic peptides, pGC = particulate guanylyl cyclase, cGMP = cyclic guanosine monophosphate, CNG = cyclic nucleotide-gated ion channels, PK-G = protein kinase G and PDE = phosphodiesterase.

Glutamate has the ability to stimulate several postsynaptic receptors, including NMDA, AMPA and kainic acid receptors, as well as numerous classes of metabotropic receptors (Carlson *et al.*, 2006). The NO pathway serves as a pivotal down-stream messenger of the glutamate-NMDA receptor signalling cascade and is of interest due to its implication in the pathophysiology of MDD. **Figure 2-17** below illustrates the Glu-NO-cGMP pathway in the mammalian brain.

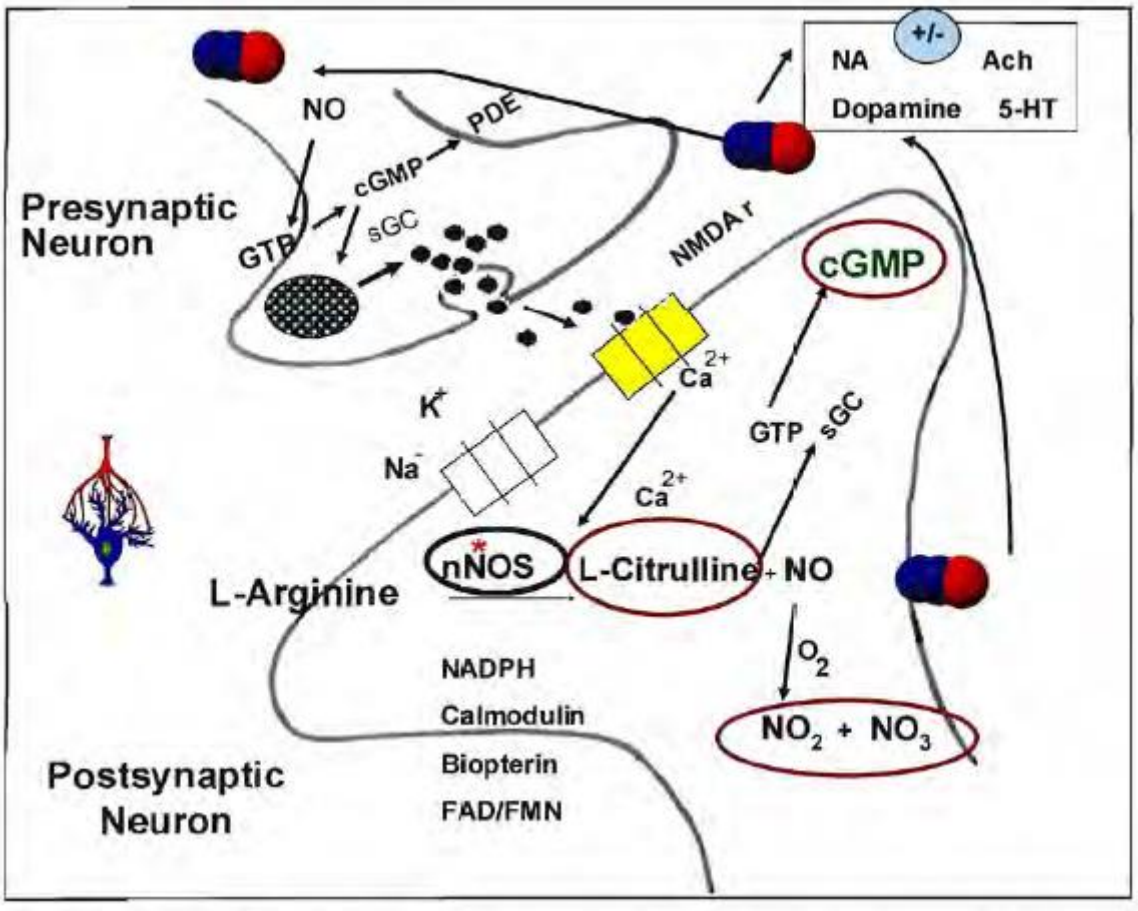

Figure 2-17: An illustration of the Glu-NO-cGMP pathway in the mammalian brain. Adapted from (Contestabile *et al.*, 2003; Ledo *et al.*, 2004). With abbreviations: NO = nitric oxide, GTP = guanosine triphosphate, cGMP = cyclic guanosine monophosphate, PDE = phosphodiesterase, sGC = soluble guanylyl cyclase, Na^+ = sodium ion, K^+ = potassium ion, Ca^{2+} = calcium ion, O_2 = molecular oxygen, NO_2 = nitrogen dioxide, NO_3 = nitrate, NMDA r = N-methyl-D-aspartate, NA = noradrenaline, Ach = acetylcholine, 5-HT = serotonin, NADPH = nicotinamide adenine dinucleotide phosphate, FAD = flavin adenine dinucleotide, FMN = flavin mononucleotide and nNOS = neuronal nitric oxide synthase.

The glutamatergic signalling cascade comprises of a sequence of events through a transduction system that takes place over a period of only parts of a millisecond (Minnaar, 2008) and the glutamatergic signalling cascade can be seen illustrated in **Figure 2-17**. Following the synthesis of glutamate from glucose and glutamine within the presynaptic neuronal terminal, glutamate is released through the process of non-constitutive exocytosis (triggered by an influx of Ca^{2+} ions) from its presynaptic effector vesicle (Belsham, 2001). Glutamate then reaches the presynaptic membrane, merges with the presynaptic membrane and is released into the synaptic cleft (exocytosis) (Roberts & Squire, 2003). Subsequently, glutamate diffuses across the synaptic cleft and acts on the postsynaptic NMDA receptors to bring about an effect (Vander *et al.*, 2001). Following glutamate's binding to the receptor binding site, the voltage-gated ion channel NMDA receptors are activated, resulting in the opening of ion channels (see **Figure**

2-17). These voltage-gated ion channels are nonselective for cations and allow the flow of sodium (Na^+) and small amounts of calcium (Ca^{2+}) ions into the cell, whereas potassium (K^+) ions are allowed to flow out of the cell (Ledo *et al.*, 2004) Glutamate's action is ended by its reuptake into the presynaptic neuronal terminal or by glutamic acid decarboxylase metabolism (Belsham, 2001).

A pivotal discovery that revolutionised the understanding surrounding synaptic neurotransmission and neuronal communication is the discovery that NO in the central nervous system mediates an elevation in the concentration of cGMP, following NMDA receptor stimulation (see **Figure 2-16** and **Figure 2-17**) (Garthwaite *et al.*, 1988a). When calmodulin, co-factors and co-substrates are present, *l*-citrulline and NO are produced from the substrate *l*-arginine through the action of enzymes known as nitric oxide synthases (NOS) (Bredt & Snyder, 1990; Prast & Philippu, 2001). The released NO subsequently activates soluble guanylate cyclase (sGC), which in turn converts guanosine-5'-triphosphate (GTP) into the second messenger cGMP (Minnaar, 2008).

Three isozymes of NOS catalyse this reaction, viz. endothelial NO synthase (eNOS), inducible NO synthase (iNOS) and neuronal NO synthase (nNOS) (see **Figure 2-16**) (Bredt & Snyder, 1990; Prast & Philippu, 2001). However, nNOS is expressed more abundantly than eNOS and iNOS in the central nervous system and can be found in several different neurons (Bredt & Snyder, 1990; Prast & Philippu, 2001). Moreover, the activation of both nNOS and eNOS requires NMDA receptor stimulation by glutamate (Friebe & Koesling, 2003). Calmodulin-dependent nNOS is activated through the influx of Ca^{2+} ions following NMDA receptor complex channel opening (Garthwaite *et al.*, 1988a) and eNOS is also a calmodulin- and Ca^{2+} influx-dependent NOS (Garthwaite *et al.*, 2006). In addition, eNOS is thought to be confined to endothelial cells, yet the NO synthesised by eNOS can diffuse to nearby neurons (Garthwaite *et al.*, 2006), whereas iNOS is not usually found in the central nervous system and is only expressed after inflammatory stimuli (MacNaul & Hutchinson, 1993).

Guanylate cyclase has both membrane-bound (particulate guanylyl cyclase (pGC)) and soluble (sGC) isoforms, with the soluble isoform found expressed mostly in the central nervous system (Dawson & Dawson, 1995; Domek-Lopacinska & Strosznajder, 2005). As previously mentioned, sGC is the main target for NO and when activated leads to an elevation in cGMP production and a subsequent activation of cGMP-dependent protein kinase (PK-G) (see **Figure 2-16**) (Friebe & Koesling, 2003). In addition, an elevation in cGMP can also be produced by natriuretic peptides (NP) through the activation of pGC (Kuhn, 2004). Furthermore, cGMP can also signal separately from PK-G through activating cyclic nucleotide-gated ion channels and/or by modulating phosphodiesterase (PDE) activity (Feil & Kleppisch, 2008).

Despite the other known signalling mechanisms of cGMP, the main signal transduction pathway of NO and cGMP involves the activation of cGMP-dependent PK-G (Hofmann *et al.*, 2009). In the brain, two subtypes of PK-G are discernible, i.e. PK-G(I) and PK-G(II) (Feil *et al.*, 2005a). PK-G(I) appears to be more abundant than PK-G(II) and is found in the hippocampus, cerebral cortex and amygdala (Feil *et al.*, 2005b). Moreover, PDEs play a pivotal part in regulating both cyclic adenosine monophosphate (cAMP) and cGMP signalling (Sonnenburg & Beavo, 1994). In this regard, cGMP is degraded through hydrolysis by both cGMP-selective PDEs (e.g. PDE5) and non-selective PDEs and the effects of cGMP are therefore terminated by PDEs (Feil & Kleppisch, 2008).

Thus, cGMP can modulate its own and/or the concentration of cAMP by inhibiting or activating a number of PDEs (Feil & Kleppisch, 2008). For instance, cGMP can elevate cAMP signalling through binding to the cGMP-inhibited cAMP-selective PDE3, whereas cGMP can decrease the concentrations of both cyclic nucleotides through binding to the cGMP-stimulated non-selective PDE2 or lower its own concentration through binding to the cGMP-selective PDE5 (Feil & Kleppisch, 2008). Therefore, cGMP-regulated PDEs play a pivotal part in regulating cyclic nucleotide signalling and can transform cGMP signals into cAMP signals, resulting in cyclic nucleotide cross-talk within the brain (Harvey *et al.*, 1990). With that said, PDE5 is expressed in several brain regions, including the cerebellum, hippocampus, caudate nucleus, substantia nigra and subthalamic nucleus (Loughney *et al.*, 1998; Van Staveren *et al.*, 2004; Menniti *et al.*, 2006).

A further degradation takes place through a negative feedback mechanism (sGC desensitization), with the concentration of degradation being determined by cGMP (Domek-Lopacinska & Strosznajder, 2005). These regulatory mechanisms of cGMP metabolism seem to be distinct in various brain regions and are affected by changes in physiological and pathological conditions (Domek-Lopacinska & Strosznajder, 2005).

The significance of the Glu-NO-cGMP pathway in the pathophysiology and treatment of MDD is apparent (Harvey & Nel, 2003; Millan, 2006), especially with reference to neuronal and brain function (Oosthuizen, 2003; Harvey *et al.*, 2004). In this regard, it appears as if cGMP is also a pivotal messenger within the cholinergic system, where cGMP may be implicated in cross-talk between the cholinergic system and various other neurotransmitter mediated pathways (De Vente *et al.*, 2000). From the above-mentioned, the role that cGMP plays in signal transduction is well-established and suggests a pivotal, yet poorly understood role in both the aetiology and treatment of MDD (Wang & Robinson, 1997). Importantly, this study focuses on the Glu-NO-cGMP-PK-G pathway (a sub-cellular signalling system) and aims to induce mood-regulatory effects (i.e. antidepressive-like effects) by modulating a downstream target of this signal

transduction pathway, i.e. inhibiting PDE5 and subsequently increasing cGMP levels in the brain.

2.8.1 The Glu-NO-cGMP-PK-G pathway and neurotransmitter release

Both endogenous and externally administered NO modulate the release of numerous neurotransmitters, viz. ACh, serotonin, noradrenaline, dopamine, excitatory amino acids and inhibitory amino acids (Prast & Philippu, 1992; Pogun *et al.*, 1994; Trabace & Kendrick, 2000; Wegener *et al.*, 2000). In this regard, NO can stimulate (Sporns & Jenkinson, 1997; Stanton *et al.*, 2005) or inhibit (Stanton *et al.*, 2001; Stanton *et al.*, 2003) the release of neurotransmitters, depending on the NO concentration and the brain region involved.

In fact, it is suggested that the release of mainly glutamate is increased through a cGMP-dependent mechanism and glutamate subsequently modulates the release of other neurotransmitters (Prast & Philippu, 2001). A model of retrograde signalling for NO within a glutamatergic synapse proposes that the release of glutamate from presynaptic terminals is induced by an action potential and that the released glutamate subsequently activates postsynaptic NMDA receptors, resulting in the production of NO (Feil & Kleppisch, 2008). Subsequently, the released NO stimulates cGMP synthesis and PK-G activation and through the phosphorylation of a variety of target proteins, can then either elevate or reduce neurotransmitter release (Feil & Kleppisch, 2008). **Figure 2-18** below illustrates retrograde NO signalling within a glutamatergic synapse.

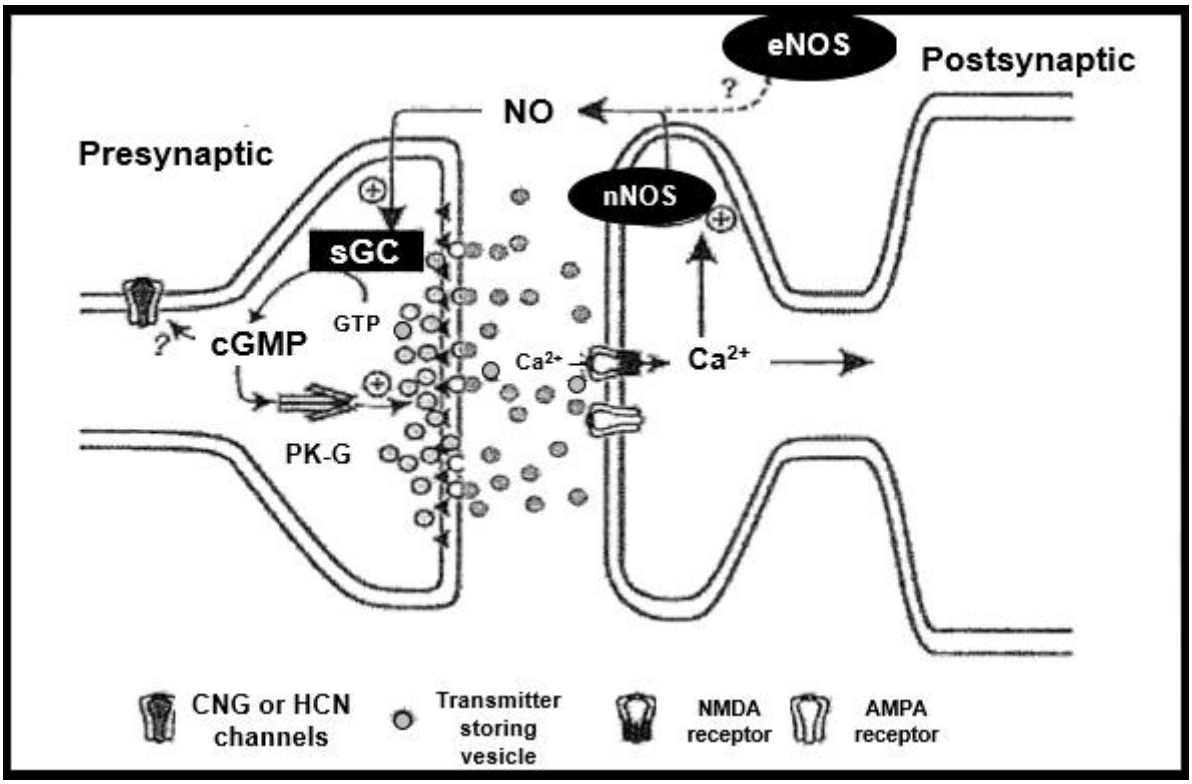

Figure 2-18: Retrograde NO signalling within a glutamatergic synapse (Feil & Kleppisch, 2008). With abbreviations: CNG = cyclic nucleotide-gated channels, HCN = hyperpolarization-activated cyclic nucleotide-gated channels, NMDA = N-methyl-D-aspartate, AMPA = alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid, cGMP = cyclic guanosine monophosphate, PK-G = protein kinase G, GTP = guanosine triphosphate, sGC = soluble guanylyl cyclase, NO = nitric oxide, Ca^{2+} = calcium ions, nNOS = neuronal nitric oxide synthase and eNOS = endothelial nitric oxide synthase.

Moreover, cGMP also plays an intricate part in modulating the presynaptic release of several neurotransmitters that are crucial for affective state, including biogenic amines (noradrenaline and dopamine), glutamate, GABA and ACh (Garthwaite, 1991; Hanbauer *et al.*, 1992; Prast & Philippu, 1992; Hirsch *et al.*, 1993; Ohkuma *et al.*, 1995; Wegener *et al.*, 2003), as well as modulating the presynaptic reuptake of neurotransmitters (e.g. serotonin) (Miller & Hoffman, 1994).

2.8.1.1 Cross-talk between the NO-cGMP pathway and the cholinergic system

There appears to be cross-talk between the NO-cGMP pathway and the cholinergic system, which will be discussed in this section. NOS inhibitors (decreasing NO synthesis) decrease ACh release *in vivo* in the basal forebrain (Prast & Philippu, 1992) and nucleus accumbens (Prast *et al*., 1998). These findings propose that cholinergic transmission in the basal forebrain and ventral striatum is tonically modulated by endogenous NO. That said, NO donors enhance ACh release in the basal forebrain (Prast and Philippu 1992) and nucleus accumbens (Prast *et al*., 1998). This facilitatory effect of NO on ACh release was also demonstrated in the medial

pontine reticular formation. In this brain region, it has been demonstrated that NOS inhibitors decrease ACh release and block the effects of neostigmine (ACh esterase inhibitor) microinjection on non-REM, sleep and breathing frequency (Leonard & Lydic, 1997). In *in vitro* studies, the NO donor hydroxylamine enhances the release of ^{3}[H]ACh in a concentration-dependent manner from hippocampal slices, after loading these hippocampal slices with the radiolabelled neurotransmitter (^{3}[H]ACh) (Lonart *et al*., 1992). In addition, NO donors also enhance the release of ACh from primary cultured cerebral cortical neurons (Ohkuma *et al*., 1995b). Therefore, a release-enhancing effect of NO was consistently demonstrated in both *in vivo* and *in vitro* studies.

Superfusion experiments have demonstrated that the release of ACh by NO donors in the basal forebrain was abolished by pre-superfusion with LY-83,583, an inhibitor of guanylyl cyclase (Prast *et al*., 1995). Since the inhibition of guanylyl cyclase may decrease cGMP levels, these data suggest that cGMP may be implicated in the NO-mediated release of ACh. In the nucleus accumbens, the release of ACh elicited by NOS donors is also abolished by guanylyl cyclase inhibitors (Prast *et al*., 1998). Microdialysis experiments have further demonstrated that the release of ACh is stimulated in a concentration-dependent manner in the striatum by the cGMP analogue 8-bromo-cGMP (Guevara-Guzman *et al*., 1994). Also, a previous *in vitro* study in our laboratories demonstrated that sildenafil (i.e. a PDE5 inhibitor, enhancing cGMP levels) can potentiate cholinergic muscarinic receptor (mAChR) function (Eager, 2004). Therefore, it is likely that the NO-induced modulation of cholinergic neurons may be mediated by cGMP (Prast & Philippu, 2001).

2.8.2 Effects of Glu-NO-cGMP-PK-G pathway modulation

Preclinical studies have demonstrated that the inhibition of the Glu-NO-cGMP-PK-G pathway through sGC inhibitors (Eroglu & Caglayan, 1997; Heiberg *et al.*, 2002; Dhir & Kulkarni, 2007; Ghasemi *et al.*, 2008) and inhibitors of NOS (Harkin *et al.*, 1999; Heiberg *et al.*, 2002; Harkin *et al.*, 2004; Dhir & Kulkarni, 2007) induces antidepressive-like behavioural responses in rodent models of MDD, whereas selective PDE5 inhibitors (e.g. sildenafil) attenuate the antidepressive-like activity of other drugs (Dhir & Kulkarni, 2007; Kulkarni & Dhir, 2007; Ghasemi *et al.*, 2008; Jesse *et al.*, 2008). Moreover, it has been demonstrated that serotonergic antidepressants inhibit NOS activity (Wegener *et al.*, 2003) and that individuals suffering from MDD present with elevated plasma nitrite levels – a product of NO metabolism (Suzuki *et al.*, 2001). Overall, these studies suggest that Glu-NO-cGMP-PK-G pathway inhibition may lead to antidepressive actions. Therefore, it was expected that PDE5 inhibition and the subsequent bolstering of the Glu-NO-cGMP-PK-G signalling pathway would lead to a worsening of depressive-like behaviour in rats (observed with an increased immobility in the FST). However, preclinical studies

demonstrated the contrary when it was discovered that sildenafil (i.e. a PDE5 inhibitor) in fact has an antidepressant-like effect in rats (Liebenberg *et al.*, 2010a). This discovery ultimately led to the working hypotheses of the current study.

As previously mentioned, preclinical studies in rodent models from our laboratories were the first to indicate that the enhancement of cGMP signalling, through selective PDE5 inhibition with sildenafil, has antidepressive-like properties at higher doses of sildenafil (≥ 10 mg/kg/day) when combined with a muscarinic ACh receptor antagonist (atropine) (Brink *et al.*, 2008; Liebenberg *et al.*, 2010a) and that the simultaneous administration of atropine is not needed for sildenafil to bring about antidepressant-like effects at a lower dose (3 mg/kg/day) (Liebenberg *et al.*, 2010a). Moreover, these antidepressant-like effects of PDE5 inhibitors (i.e. sildenafil and tadalafil) in animal models have later been confirmed by numerous other independent laboratories around the world (Baek *et al.*, 2011b; Matsushita *et al.*, 2012; Tomaz *et al.*, 2014; Wang *et al.*, 2014b; Socała *et al.*, 2016).

It is suggested that sildenafil's lack of antidepressant-like effects observed at higher concentrations, without the simultaneous administration of atropine, is due to its cholinotropic effects (i.e. depressogenic effect) in addition to its ability to elevate cGMP concentrations (i.e. antidepressant-like effect) within the central nervous system (Brink *et al.*, 2008). In this regard, the cholinotropic effects of sildenafil have been demonstrated in our laboratories in an *in vitro* cell culture study (Brink *et al.*, 2008), supported by *in vivo* preclinical studies (Devan *et al.*, 2004; Patil *et al.*, 2004). Therefore, the antidepressant-like effect of sildenafil (due to an elevation in central nervous system cGMP concentrations) is "masked" by the simultaneous elevation in cholinergic neurotransmission, so that the antidepressant-like effect of sildenafil can be "unmasked" by a centrally acting antimuscarinic agent (e.g. atropine) (Brink *et al.*, 2008).

Furthermore, clinical studies have also shown that individuals suffering from MDD present with a decreased number of NOS immunoreactive neurons (Bernstein *et al.*, 2002) and decreased NOS activity (Chrapko *et al.*, 2004; Chrapko *et al.*, 2006), supporting the notion that the bolstering of the Glu-NO-cGMP-PK-G signalling pathway results in antidepressive effects. With regards to the conflicting results obtained from preclinical studies, some studies propose that NO donors and inhibitors of NOS exhibit dual effects in rodents (i.e. antidepressive- and depressive-like effects) and that the behavioural responses are dose-dependent (Da Silva *et al.*, 2000; Inan *et al.*, 2004). Also, this is in line with the dual effects previously described for NO in the modulation of the release of numerous neurotransmitters, i.e. NO can either stimulate or inhibit neurotransmitter release and therefore subsequent neurotransmission (Feil & Kleppisch, 2008). Therefore, both depressogenic and antidepressive-like effects have been observed following modulation of the Glu-NO-cGMP-PK-G signalling pathway and the resultant effect (i.e.

depressogenic or antidepressive-like) appears to be dependent on the brain region involved, amount of stimulation and duration of treatment.

The Glu-NO-cGMP-PK-G signalling pathway is also implicated in the regulation of anxiety-like behaviour, as demonstrated by numerous preclinical studies. In this regard, exposure to NOS inhibitors induces anxiolytic-like effects in rodents, as observed in the elevated plus maze test (EPM) (Volke *et al.*, 1997; Yildiz *et al.*, 2000) and light-dark compartment test (Volke *et al.*, 2003a). Also, exposure to NO donors causes anxiolytic-like effects, as observed in the light-dark compartment test in mice (Li & Quock, 2002). However, one study has also demonstrated the contrary to the above-mentioned anxiolytic-like effects of NOS inhibitors, as anxiogenic-like effects were observed following the direct infusion of NOS inhibitors into the hippocampus or amygdala of rats and thereby inhibiting Glu-NO-cGMP-PK-G signalling (Monzon *et al.*, 2001).

Augmentation of Glu-NO-cGMP-PK-G signalling (through the acute inhibition of cGMP-selective PDE5) elevates anxiety-like behaviour in rodents, as studies on the acute effects of PDE5 inhibitors on anxiety-like behaviour have demonstrated that sildenafil elevates anxiety-like behaviour in mice (Volke *et al.*, 2003b; Volke *et al.*, 2003b; Kurt *et al.*, 2004), whereas another study has shown that *chronic* sildenafil treatment *decreases* anxiety-like behaviour in rats (Solís *et al.*, 2008). A study conducted in our laboratories has demonstrated anxiolytic-like effects in rats following sub-chronic sildenafil and tadalafil (selective PDE5 inhibitors) exposure (Liebenberg *et al.*, 2012). This pattern of response is the same as that of the SSRIs, as acute exposure to SSRIs is known to be anxiogenic, whereas chronic exposure is known for an anxiolytic response (Harvey, 1997). Thus, it seems as if the anxiolytic-like activity of PDE5 inhibitors and SSRIs (following chronic exposure) may be dependent on promoting long-lasting adaptive alterations.

Therefore, both anxiogenic- and anxiolytic-like effects have been observed in rodents following exposure to drugs that modulate NO-cGMP signalling. With that said, it appears as if the Glu-NO-cGMP-PK-G signalling pathway may be implicated in both anxiogenic- and anxiolytic-like actions within the central nervous system. Furthermore, the increase or decrease in anxiety-like behaviour following modulation of the Glu-NO-cGMP-PK-G pathway appears to be dependent on the brain region involved, dose of the modulating agent and duration of treatment.

The Glu-NO-cGMP-PK-G signalling pathway plays a significant role in the expression of synaptic plasticity that is essential for learning and memory, i.e. LTP and long-term depression (LTD) (Mize *et al.*, 1998; Ito, 2001b; Whitlock *et al.*, 2006). Pre-clinical studies have demonstrated that the inhibition of endogenous NO leads to impairment of spatial learning (Böhme *et al.*, 1993) and that rats treated with NOS inhibitors display performance impairment

in both the Morris water maze and novel object recognition test (nORT), evaluating spatial and recognition memory respectively (Chapman *et al.*, 1992; Prickaerts *et al.*, 1997; Zou *et al.*, 1998; Kirchner *et al.*, 2004). However, the contrary has also been demonstrated in rats, as the action of NO on both learning and memory appears to be cGMP-dependent and numerous of the above-mentioned responses can be replicated by exposure to a cGMP analogue (Bernabeu *et al.*, 1996) or a cGMP-selective PDE inhibitor (Prickaerts *et al.*, 1997).

However, more recent studies that have been conducted in rodents have demonstrated an improvement in learning and memory following selective PDE5 inhibition, through the stimulation of NO-cGMP signalling (Baek *et al.*, 2011b; Boccia *et al.*, 2011; Palmeri *et al.*, 2013; Jin *et al.*, 2014), whereas earlier studies demonstrated that PDE5 inhibitors enhance object recognition memory and attenuate spatial learning deficits caused by NOS inhibitors and antimuscarinic drugs in rats (Prickaerts *et al.*, 2002; Devan *et al.*, 2004; Prickaerts *et al.*, 2004; Devan *et al.*, 2006; Rutten *et al.*, 2007). These conflicting results substantiate the involvement of the Glu-NO-cGMP-PK-G signalling pathway in cognitive functioning and although this involvement is not well understood, it appears as if an elevation in NO-cGMP signalling has procognitive effects.

2.8.3 Selective PDE5 inhibitors and their neurological effects

Sildenafil (Viagra®) is the most commonly prescribed pharmacological treatment for male erectile dysfunction (Puzzo *et al.*, 2008) and was discovered during the search for novel pharmacological treatment options for pulmonary arterial hypertension and angina pectoris (Osterloh, 2004). In this regard, sildenafil is also registered for the pharmacological treatment of pulmonary arterial hypertension in adults and infants (Revatio®) (Puzzo *et al.*, 2008; Vargas-Origel *et al.*, 2010; Schwartz *et al.*, 2012). Besides its peripheral effects, sildenafil also induces a number of neurological and behavioural effects (Uthayathas *et al.*, 2007) and may therefore offer a novel approach in the treatment of psychiatric disorders, e.g. MDD.

Several drugs that selectively inhibit PDE5 (with distinct chemical classes) have been synthesised, including cGMP-based drugs (i.e. sildenafil and vardenafil) and a beta-carboline-derived drug (i.e. tadalafil) (Kim, 2003), and vardenafil is thought to be the most potent inhibitor of PDE5 (Kim, 2003). Also, sildenafil crosses the blood-brain barrier (Puzzo *et al.*, 2008) and studies have also demonstrated central nervous system effects following systemic administration of tadalafil and vardenafil in rats (Prickaerts *et al.*, 2002; Zhang *et al.*, 2006a; Ko *et al.*, 2009).

Important to note is that the paediatric use of sildenafil can have some side effects. In this regard, an overall incidence of 30% was observed between January 2011 and May 2014 for vascular, gastrointestinal and neurologic side effects in paediatric patients that received sildenafil treatment for pulmonary arterial hypertension (Siehr *et al.*, 2015). The incidence of side effects by system was 37% gastrointestinal, 35% vascular and 22% neurologic and the most commonly reported side effects of sildenafil monotherapy included: diarrhoea (26%), hyperactivity (25%), pyrexia (24%), dyspepsia (22%) and flushing (22%), however, in general, sildenafil seems to be well tolerated (Siehr *et al.*, 2015).

Studies have demonstrated that sildenafil and tadalafil elevate neurogenesis in rats (Zhang *et al.*, 2002; Zhang *et al.*, 2006a; Zhang *et al.*, 2006b) and PDE5 inhibitors also modulate mechanisms of synaptic plasticity, i.e. LTD and LTP (Puzzo *et al.*, 2008). Furthermore, PDE5 inhibitors have effects on neuronal growth modulating substances (CREB and BDNF) (Puerta *et al.*, 2010), second messenger systems (Garthwaite *et al.*, 1988b) and various other neurotransmitters within the central nervous system (Harvey *et al.*, 2006; Feil & Kleppisch, 2008; Wegener *et al.*, 2010), all known to be involved in MDD. The therapeutic potential of PDE5 inhibitors, i.e. *supporting neuroplasticity* (Zhang *et al.*, 2002; Zhang *et al.*, 2006a; Zhang *et al.*, 2006b; Puzzo *et al.*, 2008), *enhancing memory* (Ito, 2001a; Whitlock *et al.*, 2006) and exerting *anxiolytic*- and *antidepressive-like effects* (Liebenberg *et al.*, 2010a; Liebenberg *et al.*, 2012), has raised much awareness of their possible use in numerous neurological and psychiatric illnesses (e.g. MDD).

2.9 Animal models of depression

Invasive pharmacological studies are sometimes difficult or impossible to perform in humans, notably due to ethical considerations. Yet the human body represents such a complex system, that the use of only *in vitro* systems for experimentation may be too reductionistic to answer questions that can be addressed only in more complex biological systems, such as in an intact, living organism. For this reason, animal models are often used in preclinical investigations to better our understanding of the mechanisms of human diseases and pharmacokinetics and -dynamics where they have demonstrated reliable translational validity. In this regard, the use of animals allows for biological and molecular level analyses (Andersen & Navalta, 2011), or even harmful physiological and psychological impact, to better understand the underlying mechanisms of disease and treatment. In addition, animal models enable investigations to be conducted under controlled conditions (Andersen & Navalta, 2011), especially in cases where untreated controls or other strict control conditions in human studies present with extremely challenging ethical considerations, in particular research conducted in vulnerable humans, such as juveniles. Moreover, animal models can be used to investigate drug effects without the

challenge of drug-drug interactions due to multiple drug use, as is frequently observed in clinical trials (Andersen & Navalta, 2011). This is important to note, since individuals suffering from MDD often seek secondary treatment options (Andersen & Navalta, 2011).

Furthermore, the investigation of later-in-life drug effects may be more feasible in animal models, considering the shorter life cycle of animals. In this regard, a long delay exists between childhood interventions and the subsequent later-in-life effects thereof in adulthood, and results can thus only be obtained after decades, compared to weeks in some animal models (Andersen & Navalta, 2011). Since non-adherence during chronic drug-therapy is a major challenge in humans, the use of animal models may present with an enormous advantage in this regard (Andersen & Navalta, 2011). The shorter lifespan, as well as more rapid and cost-effective breeding of animals enable the use of larger numbers per treatment group, for statistically valid and repeatable results.

As mentioned above, animal models can provide us with useful insights into the basic mechanism of drug action and disease, however data should ultimately be translatable to drug action in humans or human disease (Andersen & Navalta, 2011). Animal studies have limitations and these limitations should be taken into consideration when interpreting data (Andersen & Navalta, 2011). Limitations can be attributed to inter-species differences, the complexity of neurodevelopment and the alignment of comparable developmental periods between animals and humans for comparison and therefore ensuring translatability of findings (Andersen & Navalta, 2011). However, these processes in mammals are often remarkably similar, allowing for extrapolation to humans and this includes neurodevelopment (Andersen & Navalta, 2011).

When intending to develop animal models of depression, it is worthy to consider that MDD presents itself in the form of many different characteristics and behavioural symptoms of which many cannot be induced and/or measured. Thus far, there is not one animal model of depression that is able to replicate the depressive phenotype observed in humans suffering from MDD with precision (Overstreet *et al.*, 2005; Berton & Nestler, 2006a). A number of animal models of depression have been developed and some are listed in **Table 2-8** below.

Table 2-8: A list of animal models of depression (Overstreet, 1993).

List of animal models of depression
* Congenitally learned helpless and congenitally non-learned helpless rats
* Fawn-hooded rats
* **FSL rats** (used in this study – see section 2.9.2 for a broader discussion of the FSL rat)
* High and low reaction to stress test mice
* Swim high-active and swim low-active rats
* Wistar Kyoto rats

The FSL rat (i.e. a genetic animal model of depression) was chosen for this study to investigate the role of genetic susceptibility to develop MDD in the potential later-in-life bio-behavioural and cognitive effects of early-life sildenafil treatment. At the neurobiological level, the FSL rat presents with multiple abnormalities consistent with proposed hypotheses of depression (not only cholinergic hypersensitivity as initially proposed) (Yadid *et al*., 2000). In this regard, the FSL rat displays abnormalities in both serotonergic and cholinergic function (Yadid *et al*., 2000). Importantly, serotonergic (Linthorst *et al*., 2002) and cholinergic dysfunction (Janowsky *et al*., 1972) are not only significantly implicated in the neurobiology of MDD (see section 2.5.1), but both serotonergic (Chanrion *et al*., 2007) and cholinergic function (Brink *et al*., 2008) are known to interact with the NO-cGMP signalling cascade (Wegener *et al*., 2010). Since this study is based on investigating the later-in-life bio-behavioural effects of early-life sildenafil (i.e. a drug that modulates NO-cGMP signalling) treatment, the FSL rat was chosen as an appropriate (genetic) animal model of depression for this study, with SD rats as behavioural control. A broader discussion of the FSL rat follows in section 2.9.2.

2.9.1 The validity of animal models of depression

For animal models of depression to be valid (i.e. to possess translational value for the corresponding human condition), adherence to the criteria listed and briefly described in **Table 2-9** below is required. The first three criteria listed in **Table 2-9** are considered to be the most important criteria that all animal models of depression must adhere to in order to be valid and these criteria are also the most commonly used criteria in current literature to validate animal models of MDD (Neumann *et al.*, 2011; Schmidt, 2011; Overstreet & Wegener, 2013).

Table 2-9: Criteria for the validity of animal models of depression. Adapted from (Neumann *et al.*, 2011; Schmidt, 2011; Overstreet & Wegener, 2013).

Criteria	Description of criteria
Face validity	Face validity deduces that the animal model mimics or exhibits the same symptomatology as observed in the human disorder.
Construct validity	Construct validity deduces that the animal model exhibits similar bio-pathologies that underlie MDD to humans suffering from the same disorder.
Predictive validity	Predictive validity deduces that the animal model responds to antidepressant therapy similarly to humans suffering from MDD.
Aetiological validity	Aetiological validity deduces that the animal model exhibits a pathological/genetic predisposition to develop MDD similar to some individuals suffering from MDD, e.g. an elevated genetic-environmental relation that increases the risk of developing MDD or as displayed by the enhanced cholinergic sensitivity of the FSL rat model.
Population validity	Population validity deduces that the rate of MDD incidence in an animal model population is comparable to that observed in human populations of individuals suffering from the same disorder.

With that said, there are at least eighteen valid translational animal models of depression currently in existence and these animal models can be categorised into different model types, i.e. genetic, stress, pharmacological and diverse animal models (Overstreet, 1993; Overstreet, 2002). As previously mentioned, the current study was conducted in FSL rats, i.e. a validated genetic animal model of depression (Overstreet, 1993; Overstreet, 2002). Therefore, the FSL rat will be discussed in broader detail below (see section 2.9.2).

2.9.2 The FSL rat as an animal model of depression

At first, the FSL rat model was developed from inbreeding of SD rats (El Yacoubi & Vaugeois, 2007; Hascup *et al.*, 2011) to be inherently resistant to an organophosphate and

anticholinesterase agent, diisopropyl fluorophosphate (DFP) (Overstreet *et al.*, 1979). Instead, the breeding program that was established to create these inherently DFP-resistant rats led to the creation of a rat strain completely opposite to the rat strain that was sought, and the created rat strain (later named the FSL rat) was actually more sensitive to DFP (Overstreet *et al.*, 1979). Moreover, a rat model more resistant to the effects of DFP (compared to the FSL rat model) was also developed and this animal model was named the FRL rat model, resembling control SD rats (Overstreet *et al.*, 1979).

In 1980 a breakthrough discovery was made when it was first demonstrated that the FSL rat displays depressive-like behaviour and hence could be a plausible animal model of depression. In particular, this discovery was initially based on observations of an elevated sensitivity to cholinergic agonists displayed by FSL rats, also observed in humans suffering from MDD when compared to healthy individuals (Overstreet *et al.*, 1982). This finding further correlated with the observation that FSL rats have a greater distribution and number of central nervous system muscarinic receptors (Overstreet *et al.*, 1984). These discoveries led to the conclusion that there are clear parallels in the pathophysiology of MDD between humans suffering from the disorder and the FSL rat model of depression, considering the occurrence of cholinergic supersensitivity in both FSL rats and depressed humans (Overstreet *et al.*, 1982). However, these represent only the initial findings, and below other findings to support face, predictive and construct validity will be discussed.

2.9.2.1 Behavioural features of the FSL rat

Several of the depressive symptoms and behaviours exhibited by humans suffering from MDD are also exhibited by the FSL rat, including a reduced appetite, an elevation in REM sleep and psychomotor activity retardation (Overstreet *et al.*, 2005; Overstreet & Wegener, 2013). With that said, the depressive-like behaviour exhibited by FSL rats will now be discussed.

FSL rats display an inherent elevated *immobility* in the FST compared to FRL and SD controls (Porsolt *et al.*, 1977a; Overstreet, 1993; Overstreet *et al.*, 1995; Lucki, 1997; Yadid *et al.*, 2000; Mouton, 2014; Schoeman *et al.*, 2017) and this is indicative of *behavioural despair* (Overstreet, 1993; Overstreet & Wegener, 2013). In addition, FSL rats that are subjected to early-life maternal separation display an even greater immobility during the FST compared to controls (El Khoury *et al.*, 2006). Another behavioural feature of FSL rats that is also observed in depressed humans includes abnormal hedonic responses and exposure to chronic mild stress causes a greater *anhedonic* response in FSL rats compared to controls (Pucilowski *et al.*, 1993). Also, in the sucrose preference test, FSL rats consume less sucrose compared to controls and this is indicative of *anhedonia* (Rea *et al.*, 2014). Furthermore, the FSL rat does

not demonstrate elevated anxiety-like behaviour in the EPM relative to FRL rats (Overstreet *et al.*, 1995), however FSL rats display an elevated anxiety-like behaviour in the social interaction test (Overstreet *et al.*, 2004) and in the active avoidance task (Overstreet *et al.*, 1990), indicating increased anxiety-like behaviour in the FSL rat, albeit not a robust characteristic and dependent on the specific behavioural test employed (Overstreet *et al*., 1990). Finally, deficits in cognition and memory have also been reported in the FSL rat (Gómez-Galán *et al.*, 2013), therefore in line with that reported in humans suffering from MDD.

Since not all of the depressive behaviour exhibited by humans suffering from MDD can be modelled in rodents, assessment of the translational validity of the FSL rat model cannot be based on behavioural attributes of MDD alone (Overstreet *et al.*, 2005). In this regard, neurobiological features of the FSL rat will be discussed in the following section.

2.9.2.2 Neurobiological features of the FSL rat

An elevation in Glu-NO-cGMP signalling within the hippocampus is observed in FSL rats exposed to a mild sub-chronic stressor, whereas the elevation in Glu-NO-cGMP signalling is not observed in FRL rats exposed to the same conditions, suggesting that the Glu-NO-cGMP pathway may be a genetic determinant for an increased susceptibility to develop MDD (Wegener *et al.*, 2010). As previously mentioned, humans suffering from MDD and FSL rats present with a cholinergic supersensitivity, amplifying behavioural responses to cholinergic agonists (Overstreet & Russell, 1982; Overstreet, 1993; Janowsky *et al.*, 1994; Overstreet *et al.*, 2005; Overstreet & Wegener, 2013) and the FSL rat model is therefore in accordance with the hyper-cholinergic hypothesis of MDD (see section 2.5.1.2.). Yet, the cholinergic abnormalities observed in FSL rats may not be central to the depressive-like features of this strain, as anticholinergic drugs do not induce antidepressive-like effects in FSL rats (Overstreet *et al.*, 1995) and drugs that alter cholinergic neurotransmission (e.g. DFP and lithium) do not alter the time that FSL rats spend immobile in the FST (Overstreet, 1993; Overstreet, 2002). Furthermore, this is in accordance with the inadequate efficacy of anticholinergic drugs in the pharmacological treatment of humans suffering from MDD.

Several serotonergic abnormalities also occur in FSL rats (Wallis *et al.*, 1988; Overstreet *et al.*, 1994; Zangen *et al.*, 1997). In this regard, FSL rats display a greater sensitivity to the hypothermic actions of selective $5HT_{1A}$ receptor agonists (Wallis *et al.*, 1988; Overstreet *et al.*, 1994), whereas increased levels of serotonin occur in the limbic regions of FSL rats compared to controls and these elevated serotonergic levels are reduced by chronic antidepressant treatment (Zangen *et al.*, 1997). Even though several serotonergic abnormalities are notable in FSL rats, it is not known whether these serotonergic abnormalities depict the neuropathology of

humans suffering from MDD (Overstreet *et al.*, 2005). It is challenging to compare serotonergic alterations in FSL rats with serotonergic assessments in humans suffering from MDD, considering increases, as well as decreases in serotonergic activity have been observed in clinical studies (Lesch, 1991; Masahiko *et al.*, 1991; Arango *et al.*, 1995).

Also, it has been confirmed that not only the serotonergic, but also glutamatergic and neurotrophic signalling pathways are implicated in the FSL rat model of depression and this is based on observed abnormalities within these pathways in FSL rats, similar to that seen in MDD (Overstreet *et al.*, 2005; Overstreet & Wegener, 2013). Moreover, differences in BDNF levels have been demonstrated between the FSL and FRL rat, i.e. the FSL rat presents with elevated blood BDNF levels and reduced hippocampal BDNF levels when compared to the FRL rat (Elfving *et al.*, 2010).

Recently, investigators have studied more thoroughly the cognitive impairments observed in the FSL rat to ascertain which neurological pathways may be implicated (Gómez-Galán *et al.*, 2013). As observed in humans suffering from MDD, FSL rats have a reduced hippocampal volume, which is associated with cognitive dysfunction and this is further associated with memory deficits (Gómez-Galán *et al.*, 2013). Also, the FSL rat has reduced neuro- and synaptic plasticity and emotional memory and this is further implicated in cognitive dysfunction (Eriksson *et al.*, 2012).

2.9.2.3 Translational validity of the FSL rat as an animal model of depression

In the current study, rats with a genetic susceptibility to develop MDD (FSL rats) were used and the FSL rat is a widely described and validated genetic animal model of depression (Overstreet & Wegener, 2013), whereas SD rats were used as healthy controls. The FSL rat adheres to all of the criteria (i.e. face, construct and predictive validity) necessary to make it a valid animal model of depression and this can be seen summarised in **Table 2-10** below.

Table 2-10: Criteria that the FSL rat adheres to, making it a valid translational animal model of depression. Adapted from (Overstreet, 1993; Overstreet *et al.*, 1995; Bunney & Bunney, 2000; Overstreet *et al.*, 2005; Luscher *et al.*, 2011; Neumann *et al.*, 2011; Hasselbalch *et al.*, 2012; Overstreet, 2012; Serafini, 2012; Overstreet & Wegener, 2013; Harvey & Slabbert, 2014; Haase & Brown, 2015; O'Leary *et al.*, 2015; Réus *et al.*, 2015).

Criteria	Description of how the FSL rat adheres to the criteria
Face validity	The FSL rat displays several symptoms that are comparable to those displayed by humans suffering from MDD, including: • Alterations in the ability to detect painful stimuli • *Anhedonia* • Cognitive impairment • Increased REM sleep • Inert ability to manage stress (i.e. swimming *immobility*) • Moderate anxiety-like behaviour • Reduced appetite • Reduced body weight • Reduced overall activity • Social anomalies (e.g. aggression)
Construct validity	Compared to humans suffering from MDD, the FSL rat also exhibits the following bio-pathologies: • Circadian rhythm abnormalities • Dysregulation of the monoaminergic systems • HPA axis dysregulation • Increased central glutamatergic concentrations • Increased cholinergic responsiveness • Neuropeptide Y deficiencies • Neurotrophic factor concentration and regulation abnormalities
Predictive validity	Numerous antidepressant therapies (e.g. TCAs, SSRIs, MAOIs, atypical antidepressants, benzodiazepines and electroconvulsive therapy) that have been proven to be effective in the treatment of MDD in humans have been shown to be effective in reducing depressive-like behaviour in FSL rats. Furthermore, chronic, but not acute antidepressant treatment is effective to alleviate depressive-like behaviour in the FSL rat, as commonly seen in humans.

As previously mentioned, the FSL rat displays a genetic predisposition to develop MDD similar to that observed in the human disorder (i.e. aetiological validity) (Neumann *et al.*, 2011; Schmidt, 2011; Overstreet & Wegener, 2013). However, genetic susceptibility and

environmental stressors usually co-occur in MDD and according to the gene-environment hypothesis (see section 2.5.1.1), interactions between a genetic susceptibility to develop MDD and environmental factors result in MDD (Lesch, 2004).

In conclusion, the FSL rat adheres to the criteria of face, construct and predictive validity and therefore represents a valid translational genetic animal model of depression.

2.9.3 Limiting the study to male rats only

The differences observed in depressive-like behaviour, neuromarkers of depression and antidepressive-like response between male and female rodents can be ascribed to the differences in sex hormones (Dalla *et al.*, 2010). In this regard, studies suggest that the depressive-like behaviour displayed by female rodents may differ depending on the stage of their oestrous cycle (Sfikakis *et al.*, 1978; Carrier *et al.*, 2015; Kokras *et al.*, 2015), complicating the interpretation of data obtained from behavioural studies in female rodents. Therefore, due to the possible effects that the hormonal cycles of female rats may have on their behaviour during behavioural tests, neurochemistry and antidepressive-like response, female rats were excluded from this study, ensuring that the data obtained during this study were comparable and interpreted accurately.

2.10 Screening tests for antidepressant-like activity

2.10.1 Forced swim test

Porsolt and colleagues (1977) developed the FST to detect antidepressant-like activity in rats (Porsolt *et al.*, 1977a), and later also in mice (Porsolt, 2000). The FST is the most common screening test used to identify potential antidepressants, as it is easy to use, robust (reliable between different laboratories) and able to detect antidepressant-like activity over a wide range of antidepressants (Porsolt, 2000). Due to the FST having been used in this study as a screening test for possible antidepressant-like effects of sildenafil treatment, the original and adapted version (as implemented in this study) of the FST are discussed in great detail in *Addendum A*. The FST was chosen for this study to evaluate *behavioural despair*.

2.10.2 Tail suspension test

The tail suspension test is commonly used to detect antidepressant-like activity, however it is more prominent in studies using mice compared to rats (Cryan *et al.*, 2005a). The tail suspension test is based on the observation that after initial escape-directed movements, rodents will eventually revert to an immobile posture when exposed to an inescapable

haemodynamic stressor, i.e. being hung in an uncontrollable fashion by their tail (Cryan *et al.*, 2005a).

In short, rodents are suspended by their tail for a duration of 6 min and the time spent immobile in the tail suspension test is recorded either manually or by an automated device (Steru *et al.*, 1985). The time that the rodents spend immobile during the tail suspension test is an indicator of depressive-like behaviour (Cryan *et al.*, 2005a). As with the FST, chronic exposure to antidepressants reduces the time spent immobile in the tail suspension test and promotes escape-directed behaviour (Thierry *et al.*, 1986; Cryan *et al.*, 2005a). Also, the tail suspension test has the ability to detect a broad spectrum of antidepressants, is inexpensive and easy to conduct (Steru *et al.*, 1987). Therefore, the tail suspension test has significant value in detecting antidepressant activity (Cryan *et al.*, 2005a).

2.10.3 Sucrose preference test

The sucrose preference test is based on the observation that rats in depressive-like states exhibit a decreased preference for saccharin or sucrose solutions (Willner *et al.*, 1987). Since rats have a marked preference for sweet tasting solutions, it is thought that this response is indicative of depressive-like *anhedonia* (Kokras & Dalla, 2014), which is also reported by individuals suffering from MDD (Willner *et al.*, 1987). The reduction in sucrose consumption is reversed by chronic (i.e. 14 to 28 days) antidepressant treatment (Willner *et al.*, 1987). Importantly, the reduction in sucrose preference displayed by FSL rats in the sucrose preference test seems to be dependent on prior exposure to chronic mild stress, a procedure that induces a reduction in sweet intake (or anhedonia) (Pucilowski *et al.*, 1993; Willner, 2005).

In short, rats are offered a free choice between two 200 ml bottles for 24 hours, one containing 0,8% sucrose solution and the other tap water. To prevent possible effects of side preference in drinking, the bottles are switched after 12 hours. The rats are not deprived of water or food prior to the test. The consumption of water and sucrose solution is measured by weighing the bottles after the test is completed. The preference for sucrose is calculated from the amount of sucrose solution consumed, expressed as a percentage of the total amount of liquid consumed over the 24 hour test period (Rygula *et al.*, 2005).

2.11 Synopsis

Despite extensive research into the neurobiological basis of MDD and antidepressant action, an array of questions has yet to be answered. MDD is a debilitating disorder not only affecting adults but is frequently seen in children and adolescents (Bylund & reed, 2007b). Importantly,

only two SSRIs have been approved for the treatment of MDD in children and adolescents, namely fluoxetine (7–18 years) and escitalopram (12–18 years) (Oberlander & Miller, 2011; Soutullo & Figueroa-Quintana, 2013). SSRIs are more effective in children and adolescents due to the serotonergic system maturing much earlier than the noradrenergic system, suggesting a difference in neurobiology to that of adults (Murrin et al., 2007). Also, very few studies have studied the effects of psychotropic drugs on neurodevelopment, therefore it is unclear if early-life exposure to psychotropic drugs has harmful or beneficial long-lasting effects, if any.

Moreover, the Glu-NO-cGMP-PK-G pathway may have potential as a novel target for antidepressant development. Selective PDE5 inhibitors (e.g. sildenafil) appear to hold a lot of promise as potential drug candidates, that target the Glu-NO-cGMP-PK-G system, in the treatment of psychiatric disorders and it is the intent of this study to show that a selective PDE5 inhibitor (i.e. sildenafil) may induce later-in-life antidepressive-like effects into adulthood when administered during early-life.

Therefore, this study was conducted to shed some light on a few questions in this regard. Firstly, in a translational genetic animal model of MDD (i.e. the FSL rat), it was investigated whether early-life sub-chronic exposure to the psychotropic drug, sildenafil (PDE5 inhibitor), as compared to vehicle control, induces any later-in-life bio-behavioural effects as displayed in adulthood, i.e. modulation of depressive-like behaviour, cognition and neurobiological indicators of neuroplasticity and MDD. Secondly, the role of genetic vulnerability in any bio-behavioural effects of sildenafil was examined, by comparing sildenafil's effects in FSL rats (stress-sensitive) to those observed in SD rats (control). Thirdly, the role of juvenile age of sildenafil exposure on any later-in-life bio-behavioural effects was examined, by comparing sildenafil's effects after sub-chronic exposure during pre-puberty (PnD 21 - 34) to the effects observed after sub-chronic exposure during puberty (PnD 35 - 48).

Chapter 3. Article

This dissertation is written and presented in the North-West University approved *article format*. Therefore, this chapter (i.e. *Chapter 3*) provides key data in the form of a research article in a selected and suitable scientific journal. Thus, an article titled: "*Later-in-life behavioural effects of early-life sildenafil treatment in stress-sensitive versus healthy control rats*" is prepared to be submitted to the **European Journal of Pharmacology** as a complete research report.

Importantly, this chapter, unlike the other chapters of this dissertation, adheres to and was prepared according to the instructions to the author applicable to the selected journal. The instructions to the author can be viewed at https://www.elsevier.com/journals/european-journal-of-pharmacology/0014-2999/guide-for-authors. A noteworthy distinction is that the reference list of publications cited in *Chapter 3* is provided at the end of the article (i.e. *Chapter 3*) and not at the end of the dissertation as is the case for the other chapters. These specific references are also presented according to the guidelines of the scientific journal and therefore differ from that of the rest of the dissertation. In addition, although tables and figures will be submitted to the scientific journal separately, all tables and figures have been included in the text of *Chapter 3* to ease reading. Lastly, only data obtained from behavioural tests are discussed in *Chapter 3*, whereas data obtained from neurochemical analyses are discussed in *Addendum B*.

Author contributions

Juandré Lambertus Bernardus Saayman conducted all the behavioural tests, data work-up and statistical analyses of study data, assisted with data interpretation and wrote the first draft of the manuscript.

Stephanus Frederik Steyn assisted with data interpretation, provided guidance with regards to the statistical analyses of data and proofread the final manuscript.

Christiaan Beyers Brink designed, planned and supervised the study, obtained funding for the study, assisted with data interpretation and is corresponding author of the final manuscript.

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The Examiner
MSc Dissertation of JLB Saayman

22 November 2018

Dear Examiner

Permission to Mr JLB Saayman to Include Manuscript in his Dissertation for Examination Purposes for a MSc Degree

As study supervisor and senior corresponding author on a manuscript that will be submitted to an accredited international journal to be published in the near future, understanding that it was co-authored by Mr Juandré LB Saayman, I hereby approve that the manuscript be included in Chapter 3 as part of the requirements for the fulfilment of the MSc degree, and that this manuscript be submitted for examination of Mr Saayman's dissertation.

The title of the article is as follows:

Later-in-life behavioural effects of early-life sildenafil treatment in stress-sensitive versus healthy control rats

To be submitted to the European Journal of Pharmacology

I trust that you will find this in order.

Yours sincerely

Digitally signed by Christiaan B Brink
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Later-in-life behavioural effects of early-life sildenafil treatment in stress-sensitive versus healthy control rats

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Abstract

Juvenile depression is a significant global concern, yet only fluoxetine and escitalopram are approved drug treatment options. Reports suggest an increase in the prevalence of juvenile depression and concomitant antidepressant prescription rates. Current juvenile antidepressant treatment is generally associated with an initial increased risk of suicidal ideation, delayed onset of action, high relapse and low efficacy and remission rates. This highlights the need for investigations into novel antidepressant treatment strategies and drug targets, with potentially beneficial later-in-life effects as observed in adulthood. The current study investigated the later-in-life effects of sub-chronic pre-pubertal and pubertal sildenafil treatment on parameters of depressive- and anxiety-like behaviour and cognition in adult rats. Male Flinders Sensitive Line (FSL) and Sprague-Dawley (SD) rats were treated with either sildenafil (3 mg/kg/day) or vehicle-control through subcutaneous injection from postnatal day 21 to 34 (pre-pubertal period when only the serotonergic system has matured) or from postnatal day 35 to 48 (pubertal period when both the serotonergic and noradrenergic systems have matured). On postnatal day 60 (i.e. adulthood) the open field test, forced swim test and novel object recognition test were conducted to assess locomotor activity and anxiety-like behaviour, antidepressive-like behaviour and cognition, respectively. Sildenafil treatment had no effect on locomotor activity or anxiety-like behaviour in either rat strain and regardless of age during treatment. The FSL rats displayed greater immobility in the forced swim test compared to the SD rats, which was

reduced by sildenafil treatment, regardless of age during treatment. However, sildenafil treatment had no effect on the immobility of SD rats, regardless of age during treatment. In addition, sildenafil increased the time spent swimming (i.e. enhanced serotonergic neurotransmission) in pre-pubertal but not pubertal treated rats (i.e. differences in treatment age susceptibility), regardless of the strain. Sildenafil treatment had no effect on cognition in either rat strain and regardless of age during treatment. Therefore, early-life sub-chronic sildenafil treatment has later-in-life antidepressive-like effects as observed in adulthood, but with no effect on anxiety-like behaviour and cognition.

Keywords

Depression; juvenile; sildenafil; Flinders Sensitive Line rat; cyclic guanosine monophosphate; later-in-life effects.

3.1 Introduction

Juvenile depression is a tremendous concern worldwide and is reported to be the most common psychiatric disorder affecting children (Bylund & Reed, 2007). It is estimated that 25% of juveniles will experience a major depressive episode before reaching adulthood (Kessler *et al*., 2001). Major depressive disorder (MDD) affects an estimated 10% of adolescents, 2.8% of pre-adolescents and 0.3% of pre-schoolers (Bhatia & Bhatia, 2007; Kozisek *et al*., 2008), leading to an increase in missed school days and worsening of academic performance (Owens *et al*., 2012), altogether contributing to the significant economic burden of juvenile MDD (Olesen *et al*., 2012). In addition to a delayed onset of action and low efficacy rates of antidepressant treatment in juveniles suffering from MDD (Artigas, 2017), relapse during adulthood is also a major concern (Bhatia & Bhatia, 2007). Furthermore, juvenile MDD has also been associated with memory impairments (Günther *et al*., 2004), low self-esteem (Renouf *et al*., 1997), elevated risks for suicidal behaviours (Fava & Kendler, 2000) and substance abuse (Lubman *et al*., 2007) and enduring psychosocial impairment into adulthood (Weir *et al*., 2012).

The symptoms and diagnostic criteria of MDD, across all age groups, are similar, except for irritability being a key symptom used for the diagnosis of MDD in juvenile patients (American Psychiatric Association, 2013). There has been an increase in the number of juveniles diagnosed with MDD and an associated increase in prescription rates for antidepressants to treat juvenile MDD (Bachmann *et al*., 2016). However, juvenile patients have notably less

pharmacological treatment options available compared to adults, as only two selective serotonin reuptake inhibitors (SSRIs) have been approved for the treatment of juvenile MDD. In this regard, the Food and Drug Administration (FDA) has only approved fluoxetine to treat MDD in children 7-12 years of age and fluoxetine or escitalopram to treat MDD in adolescents 12-18 years of age (Soutullo & Figueroa-Quintana, 2013). With that said, the FDA has issued a "black-box" warning of an initial increase in suicidal behaviour with the onset of SSRI treatment in juvenile MDD patients (Friedman & Leon, 2007). Therefore, research into novel antidepressant strategies and drug targets for the treatment of juvenile MDD, with potentially beneficial later-in-life outcomes, is urgently warranted.

The Flinders Sensitive Line (FSL) rat is a validated genetic animal model of MDD, presenting with face, construct and predictive validity (Overstreet *et al*., 2005; Overstreet & Wegener, 2013). These animals display exaggerated immobility in the forced swim test (FST), a screening procedure for depressive-like behaviour in rodents (Porsolt, 1979; Porsolt *et al*., 1979), and respond to chronic but not acute antidepressant treatment when examined in the FST (Overstreet *et al*., 2005; Yadid *et al*., 2000). The FSL rat was used in this study to investigate the role of genetic susceptibility to develop MDD in the possible later-in-life behavioural and cognitive effects of early-life sub-chronic sildenafil treatment.

Although the FSL rat was initially thought to be a hypercholinergic model, it was later discovered that the FSL rat presents with multiple neurobiological abnormalities (Yadid *et al*., 2000). For instance, in addition to abnormalities in cholinergic function, the FSL rat also displays abnormalities in serotonergic function (Yadid *et al*., 2000). Importantly, serotonergic (Linthorst *et al*., 2002) and cholinergic dysfunction (Janowsky *et al*., 1972) are significantly implicated in the neurobiology of MDD and both serotonergic (Chanrion *et al*., 2007) and cholinergic function (Brink *et al*., 2008) interact with the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) signalling cascade. As a result, The FSL rat was selected as an appropriate genetic animal model of MDD for this study, since this study investigated the later-in-life behavioural and cognitive effects of early-life sub-chronic sildenafil treatment, i.e. a drug that modulates NO-cGMP signalling and ultimately enhances central cGMP concentrations. Sprague-Dawley (SD) rats were used as behavioural control in this study.

A previous study conducted in our laboratories on the phosphodiesterase type 5 (PDE5) inhibitor sildenafil demonstrated antidepressant-like effects in adult rodents (Liebenberg *et al*., 2010), whereas other studies have shown that sildenafil increases neuroplasticity (Puzzo *et al*., 2008) and enhances memory (Whitlock *et al*., 2006). This is of importance since MDD is associated with reduced concentrations of neurotrophic factors (e.g. brain-derived neurotrophic factor) and reduced neuroplasticity (Schmidt & Duman, 2007; Dwivedi, 2009). Importantly, the

glutamate-nitric oxide-cyclic guanosine monophosphate-protein kinase-G (Glu-NO-cGMP-PK-G) signalling pathway, which is modulated by sildenafil, plays a significant role in the expression of neuroplasticity, which is essential for learning and memory, i.e. long-term potentiation and long-term depression (Mize *et al.*, 1998; Ito, 2001; Whitlock *et al.*, 2006).

This study aimed to investigate the later-in-life effects of early-life sildenafil treatment on depressive- and anxiety-like behaviour and cognition as observed in adult rats. In addition, this study also investigated the role of genetic susceptibility to develop MDD, by comparing the results obtained from a genetic animal model of MDD, i.e. the FSL rat, to results obtained from a control line, i.e. the SD rat. Finally, this study investigated the age at which sildenafil treatment could potentially induce the most robust later-in-life antidepressant-, anxiolytic-like and pro-cognitive effects, i.e. during pre-puberty, puberty or both.

3.2 Materials and methods

3.2.1 Test subjects and treatment strategies

3.2.1.1 Animals

FSL rats and a comparable behavioural control line, i.e. SD rats, were used in this study. The rats were bred, supplied and housed at the Vivarium (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019; AAALAC accreditation international file #1717) of the Pre-Clinical Drug Development Platform (PCDDP) of the North-West University, RSA. The original rat colonies were obtained from Dr. David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA. The rats were group-housed 2-3 rats per cage in 395 x 346 x 213 mm (*w x d x h*) polysulphone individually ventilated cages, under conditions of constant temperature (22 ± 1°C) and humidity (55 ± 10%). A 12-hour light/dark cycle (lights on between 06:00 and 18:00) was maintained and food and tap water were provided *ad libitum*. Cages were cleaned and bedding (chipped corncob) replaced weekly.

The period between postnatal day (PnD) 21 and PnD 34 represents pre-puberty in rodents when the serotonergic pathways in the brain have already matured, whereas the noradrenergic and dopaminergic pathways are still developing (Panksepp, 2004; Murrin *et al*., 2007). With that said, the period between PnD 35 and PnD 48 represents puberty in rodents when serotonergic, noradrenergic and dopaminergic pathways have reached maturity (Murrin *et al*., 2007; Eiland & Romeo, 2013). Rat pups were therefore weaned on PnD 21 and divided into pre-pubertal (PnD 21 – 34) and pubertal (PnD 35 – 48) treatment groups. The pre-pubertal and pubertal treatment groups subsequently consisted of two cohorts each, i.e. an FSL and SD rat

group. The SD and FSL rat groups were further subdivided into two groups each comprising of 12 rats, receiving vehicle control (i.e. saline) and sildenafil, respectively. After sildenafil or vehicle control treatment, rats were housed under standard laboratory conditions for a "wash-out" period until PnD 60 (i.e. adulthood) when behavioural tests were conducted. See Figure 3-1 below for a schematic illustration of the study layout.

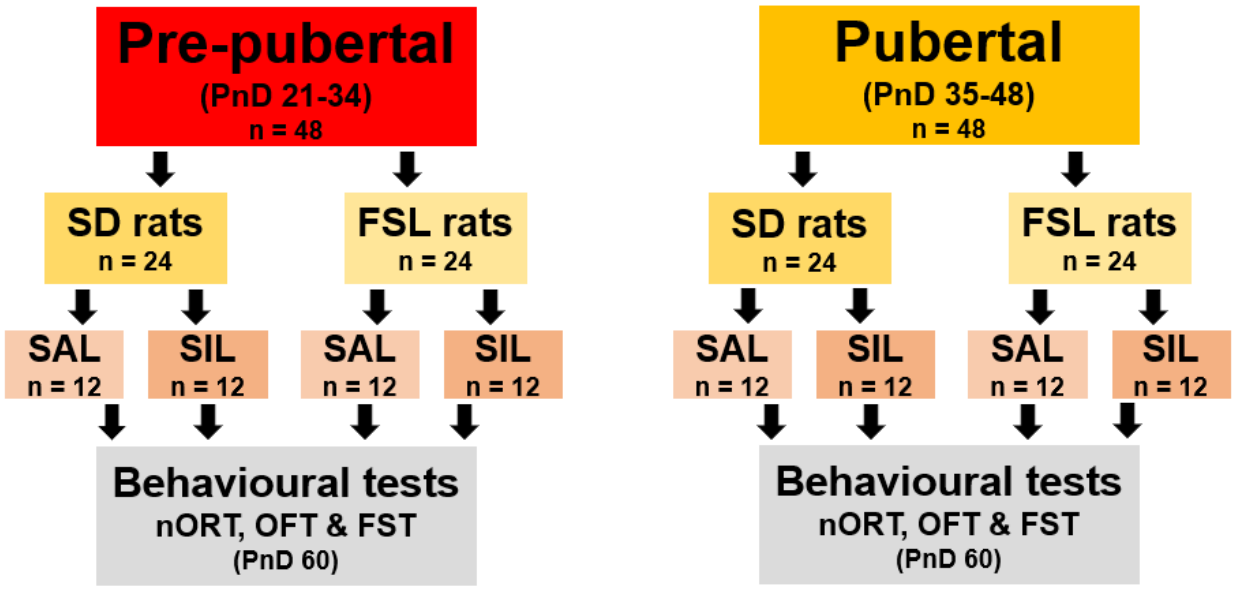

Figure 3-1: Schematic illustration of the study layout. With abbreviations: PnD = postnatal day, n = number of rats, SD = Sprague-Dawley rats, FSL = Flinders Sensitive Line rats, SAL = saline, SIL = sildenafil, nORT = novel object recognition test, OFT = open field test and FST = forced swim test.

The rats were subsequently euthanised by decapitation within 24 hours after the completion of the behavioural tests.

3.2.1.2 Drug treatment

Animals received either saline (vehicle control) or sildenafil citrate (3 mg/kg/day) (purchased from Sigma Aldrich) dissolved in saline via subcutaneous (sc) injection, once daily, between 07:00 and 10:00, from PnD 21 to PnD 34 for the pre-pubertal groups and from PnD 35 to PnD 48 for the pubertal groups. A 14-day treatment period was implemented in the current study, which is regarded as sub-chronic (Overstreet, 2002), yet sufficient to induce antidepressive-like effects in FSL rats (Overstreet, 2002; Steyn, 2011; Schoeman *et al*., 2017).

A dose of 3 mg/kg/day sildenafil citrate has previously been shown to be effective in inducing antidepressant-like effects in adult FSL rats, without requiring co-administration of an anticholinergic agent, and was therefore used in the current study (Liebenberg *et al*., 2010). Rats were weighed every day and the appropriate dose was calculated accordingly for each rat.

The rats were injected with a total volume of up to 0.2 ml to limit discomfort that might be caused by injecting a large volume into young rats. Injections via the sc route have a predictable bioavailability comparable to that of intraperitoneal injections, however injection stress is less (Schoeman *et al*., 2017), and this is particularly important in young rats. Therefore, the sc route of drug administration was used in this study, which is in line with previous studies conducted in our laboratories (Badenhorst *et al*., 2017; Schoeman *et al*., 2017).

3.2.2 Behavioural tests

As previously mentioned, following sub-chronic saline or sildenafil treatment during pre-puberty (i.e. PnD 21–34) and puberty (i.e. PnD 35–48), the rats were housed under standard laboratory conditions for a "wash-out" period until PnD 60, which represents adulthood (Tirelli *et al*., 2003; Panksepp, 2004; Malkesman & Weller, 2009). On PnD 60, all the behavioural and memory tests described below were conducted sequentially, starting with the least stressful tests and ending with the most stressful test (Mokoena *et al*., 2015). A previous study in our laboratories demonstrated that foregoing tests do not influence the outcome of subsequent consecutive tests when tests are conducted from least to most stressful (Mokoena *et al*., 2015). The behavioural tests were conducted within 6 hours from the start of the dark cycle (i.e. 18:00), implementing the novel object recognition test (nORT), open field test (OFT) and forced swim test (FST), in this order (Mokoena *et al*., 2015). Also, behavioural testing commenced 1 hour after the start of the dark cycle (i.e. 19:00) to provide accommodation for initial foraging and activity of the nocturnal animals. The behavioural tests were spaced in such a manner as to allow 1 hour between each test for habituation of the animals to the environment (behavioural testing rooms).

3.2.2.1 Novel object recognition test

Since patients who suffer from MDD frequently present with impaired declarative memory (Deuschle et al., 2004), also commonly observed in the FSL rat (Gómez-Galán et al., 2013), the nORT was implemented in this study to assess the potential later-in-life effects of early-life sub-chronic sildenafil treatment on short-term and declarative memory, i.e. a valuable measure of cognition (Antunes & Biala, 2012), as observed in adult rats. In this regard, sildenafil treatment has been shown in a previous study to enhance memory (Whitlock *et al*., 2006).

The nORT is used to measure declarative memory (i.e. a parameter of cognitive functioning) in rats and is based on the observation that rats prefer exploring novel objects over familiar objects (Bevins & Besheer, 2006; Winters *et al*., 2008). The nORT was conducted in a 1 m^2

square arena with opaque black, vertical walls (h = 45 cm) under red light (80 lx) and as previously described (Abildgaard *et al*., 2011). In short, each test comprised of an acquisition and retention trial. The acquisition and retention trial were spaced 90 min apart and the arena was wiped clean with 10% ethanol after every trial to eliminate any olfactory cues in subsequent trials. In each trial the rats were placed in the centre of the arena facing one of two immovable objects placed in two corners, 25 cm from the walls of the arena. The rats were allowed to explore the objects for 5 min and their exploratory behaviour was video-recorded with a video camera situated directly above the arena. Object exploration was defined as the rat actively sniffing, licking or physically touching the object (Abildgaard *et al*., 2011) and the video recordings were subsequently scored accordingly using Ethovision XT14 software (Noldus Information Technology BV, Wageningen, NLD).

Importantly, in the acquisition trial, the rats were allowed to freely explore two identical objects (yellow plastic ducks). After 90 min, during the retention trial, one of the objects used in the acquisition trial was replaced with a novel object (purple glass owl salt pot). Rats tend to spend more time exploring novel objects when their memory is unimpaired. With that said, the time rats spend exploring a novel object relative to a familiar object is indicative of memory consolidation. The total time that the rats spent exploring the familiar object (T1), novel object (T2) and the familiar and novel objects combined (T1 + T2) were measured. Then, the preference index (PI) was calculated for each rat by using the formula PI = $\frac{T2}{T1+T2}$ X 100 (Wang *et al*., 2007), where preference for the novel object is indicated by values greater than 50%, preference for the familiar object is indicated by values less than 50% and a value of 50% indicates no preference for the familiar or novel object (Hammond *et al*., 2004).

3.2.2.2 Open field test

Due to the high prevalence of co-morbid anxiety disorders associated with MDD (Pollack, 2005), the OFT was implemented in this study to assess the potential later-in-life effects of early-life sub-chronic sildenafil treatment on anxiety-like behaviour, as observed in adult rats. In this regard, anxiolytic-like effects of sildenafil treatment in FSL rats have been demonstrated in a previous study conducted in our laboratories (Liebenberg *et al*., 2012). The OFT is also commonly performed to evaluate locomotor activity (i.e. a parameter of the general ability of the rat to move and negotiate its environment) in addition to anxiety-like behaviour (Prut & Belzung, 2003; Hiroi *et al*., 2006; Hiroi & Neumaier, 2006). The apparatus used for the OFT in this study consisted of a 1 m^2 square test arena with opaque black, vertical walls (*h = 45 cm*). The OFT was performed as described previously for our laboratories (Schoeman *et al*., 2017; Steyn, 2018).

In short, following a habituation period of 1 hour, rats were individually placed in the centre of the OFT arena and allowed to explore the arena for 5 min under red light (80 lx) (Schoeman *et al*., 2017; Steyn, 2018). For the duration of the OFT, rats were video-recorded by a video camera mounted directly above the OFT arena and the video recordings were subsequently scored using Ethovision XT14 software (Noldus Information Technology BV, Wageningen, NLD). The total distance moved during the test was used as a measure of general locomotor activity and the total time spent in the centre zone (50 m^2) of the OFT arena was used as a measure of anxiety-like behaviour, with a reduction indicating elevated anxiety-like behaviour.

3.2.2.3 Forced swim test

The FST is commonly used to screen for antidepressive-like effects in rodents and by implication discerns between depressive- and antidepressive-like behaviour (Porsolt *et al.*, 1977). The FSL rat is a validated genetic animal model of MDD that presents with an elevated immobility in the FST (i.e. increased depressive-like behaviour), without requiring a pre-conditioning swim trial 24 hours before the testing swim trial (Overstreet *et al*., 2005). Importantly, both SD and FSL rats did not have a pre-conditioning swim trial 24 hours before the testing swim trial in the current study. This was to ensure that the behaviour of the SD rats in the testing swim trial can be compared to that of the FSL rats, without the SD rats being pre-conditioned to the swim stress of the FST, which may influence their behaviour. Furthermore, the adapted version of the FST, as implemented in this study, can discern between swimming and struggling behaviour, i.e. serotonergic and noradrenergic-directed behaviour, respectively (Lucki, 1997; Cryan & Lucki, 2000; Cryan *et al*., 2002; Cryan *et al*., 2005).

The apparatus used for the FST in this study comprised of four Perspex® cylindrical tanks ((40 cm (*h*) x 20 cm (*d*)), each filled with water to a depth of 30 cm, with the water maintained at 25 ± 1°C. The FST was conducted as described previously for our laboratories (Schoeman *et al*., 2017; Steyn, 2018). In short, following a habituation period of 1 hour, the FST was conducted during the dark cycle (i.e. after 18:00) under red light (80 lx). On the day of the FST, rats were individually placed into Perspex® cylinders filled with water and their behaviour was recorded with a video camera situated in front of the Perspex® cylinders for 5 min. The video recordings were subsequently analysed by an investigator blind to the different test groups (Slattery & Cryan, 2012).

Behaviour during the FST was scored by making use of a manual continuous timer software (FST Scoreboard 2.0 software; Academic Support Services: Information Technology in Education, NWU, RSA). This software has been validated against the 5 sec time-sampling technique in our laboratories (Badenhorst *et al*., 2017). Scored behaviour included immobility

(i.e. no active movements, except those required for the rat to keep its head above the water), swimming (i.e. horizontal movements whereby the rat crosses from one quadrant to another throughout the Perspex® cylinder) and struggling (i.e. upward-directed movements of the rat's forepaws along the inside of the Perspex® cylinder) (Cryan *et al*., 2002; Cryan *et al*., 2005). Of note, although the total time spent diving was also video-recorded during the FST, this specific behaviour was not incorporated into the final depressive-like behaviour analysis of the rats. This is due to diving behaviour being episodic and seemingly not correlating with any specific treatment effects (Cryan *et al*. 2005).

3.3 Statistical analyses

The minimum number of animals needed for statistically significant results were used, as estimated by an evidence-based estimation (Liebenberg et al., 2010). Taking $\alpha = 0.05$ as significant, the Grubbs' test was performed to identify any outlier in each data set. In this regard, the experimental group size for every data set is indicated in the results (Kilkenny *et al*., 2010). However, it was seldom necessary to exclude any data points and if in fact any data points were excluded, this was clearly indicated in the table and figure legends.

Three-way ANOVAs (analysis of variance) were performed on all data sets to determine whether statistically significant three-way interactions existed between the drug (i.e. saline or sildenafil), age of treatment (i.e. pre-pubertal or pubertal) and rat strain (i.e. FSL or SD). Where a significant three-way interaction was identified, simple two-way interactions were analysed, followed by analysis of significant simple simple main effects and significant simple simple comparisons (i.e. post-hoc test). However, in instances, where no significant three-way interactions were identified, analyses for significant two-way interactions were performed, followed by analyses for significant simple main effects and significant pairwise comparisons. In all instances, the Tukey post-hoc test was performed for multiple comparison analyses. Importantly, where more than one statistically significant two-way interactions were identified, the partial eta squared (η^2) was used to interpret the robustness and practical significance of the two or more two-way interactions. The partial eta squared is an effect magnitude indicator and accepted as a large effect size when $\eta^2 \geq 0.14$ (Ellis, 2010). In all instances, only large effect sizes were accepted as significant and robust.

GraphPad Prism® (version 7.0, San Diego California, USA) and IBM® SPSS® Statistics (version 25.0. Armonk, NY: IBM Corp), together with Laerd Statistics® (https://statistics.laerd.com), were used for both the statistical analyses and graphical representations. Data are presented as mean ± SEM, with a *p*-value of < 0.05 accepted as statistically significant.

3.4 Results

In this study, the independent variables included the rat strain (SD vs FSL), the treatment age (pre-pubertal vs pubertal) and the drug treatment (vehicle vs sildenafil). As explained above, the outcome of three-way and two-way ANOVA analyses of data sets (e.g. when three-way indicated no interaction, and two-way indicated interaction between independent variables) dictated when and how data from two groups were pooled. Accordingly, some graphs would reflect only two of the three independent variables. Also, only when post-hoc analyses were possible, differences have been indicated on the graphs, whereas differences from ANOVA analyses are discussed in the text.

For Figure 3-2, Figure 3-4 and Figure 3-5, there were no statistically significant three-way interactions between Strain (SD and FSL), Age (Pre-pubertal and Pubertal) and Treatment (Saline and Sildenafil), nor any statistically significant two-way interactions between Strain, Age or Treatment. In this regard, Figure 3-2 is displayed with [Strain] on the X-axis, sub-divided by Age (Pre-pubertal vs Pubertal). The bars are separated by Treatment (SAL or SIL). Figure 3-4 is also displayed with [Strain] on the X-axis, sub-divided by Age (Pre-pubertal vs Pubertal). The bars are separated by Treatment (SAL or SIL), similar to Figure 3-2. Figure 3-5 is displayed with [Treatment] on the X-axis, sub-divided by Strain (SD vs FSL). The bars are separated by Age (Pre-pubertal or Pubertal).

For Figure 3-3(A), Figure 3-3(B) and Figure 3-3(C), there were no statistically significant three-way interactions between Strain (SD and FSL), Age (Pre-pubertal and Pubertal) and Treatment (Saline and Sildenafil), however there were statistically significant two-way interactions between Strain, Age or Treatment. In this regard, for Figure 3-3(A) and Figure 3-3(B), Age (Pre-pubertal and Pubertal) was combined, to perform analysis after two-way analyses. However, for Figure 3-3(C), Strain (SD and FSL) was combined, to perform analysis after two-way analyses. This is the correct way to analyse data, as confirmed by the consulting statistician (and other before).

3.4.1 General locomotor activity

Figure 3-2 below depicts the later-in-life effects of early-life vehicle control or sildenafil treatment on the general locomotor activity of SD and FSL rats on PnD 60.

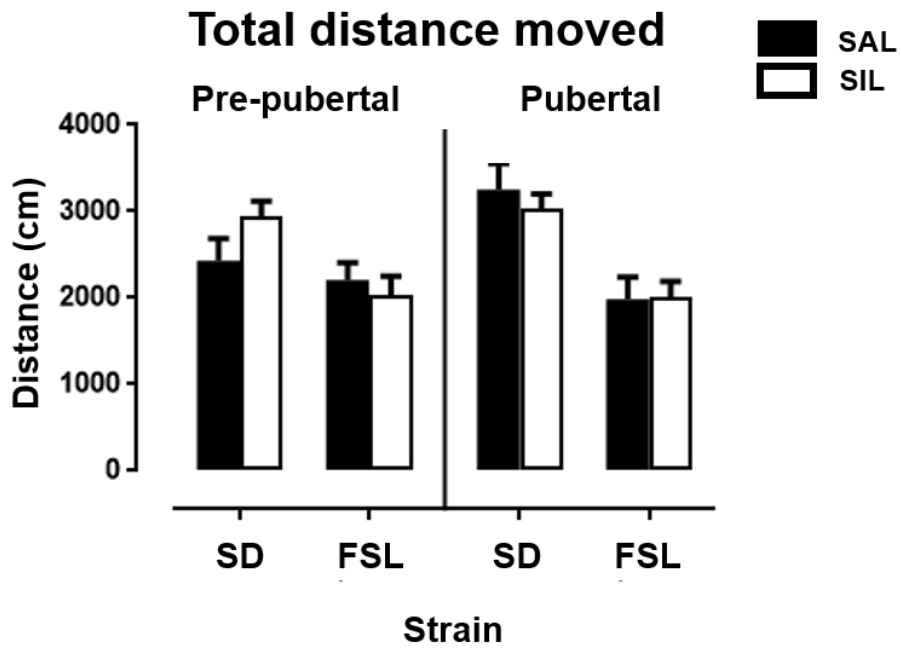

Figure 3-2: Effects of sub-chronic pre-pubertal (PnD 21–34) or pubertal (PnD 35–48) vehicle control or sildenafil treatment on the locomotor activity of SD and FSL rats in early adulthood (PnD 60). Distance moved in the OFT on PnD 60 following treatment of SAL+SD (n = 24), SAL+FSL (n = 24), SIL+SD (n = 24) or SIL+FSL (24). Data points represent the mean ± SEM. With abbreviations: SAL = saline, SIL = sildenafil, FSL = Flinders Sensitive Line rats and SD = Sprague-Dawley rats.

In Figure 3-2, there was no statistically significant three-way interaction between treatment, age and strain ($F_{1.88}$ = 2.076, p = 0.153, η^2 = 0.023) for distance moved on PnD 60, nor any statistically significant two-way interactions.

3.4.2 Depressive-like behaviour

Figure 3-3 below depicts the later-in-life behavioural effects of early-life vehicle control or sildenafil treatment on SD and FSL rats in the FST.

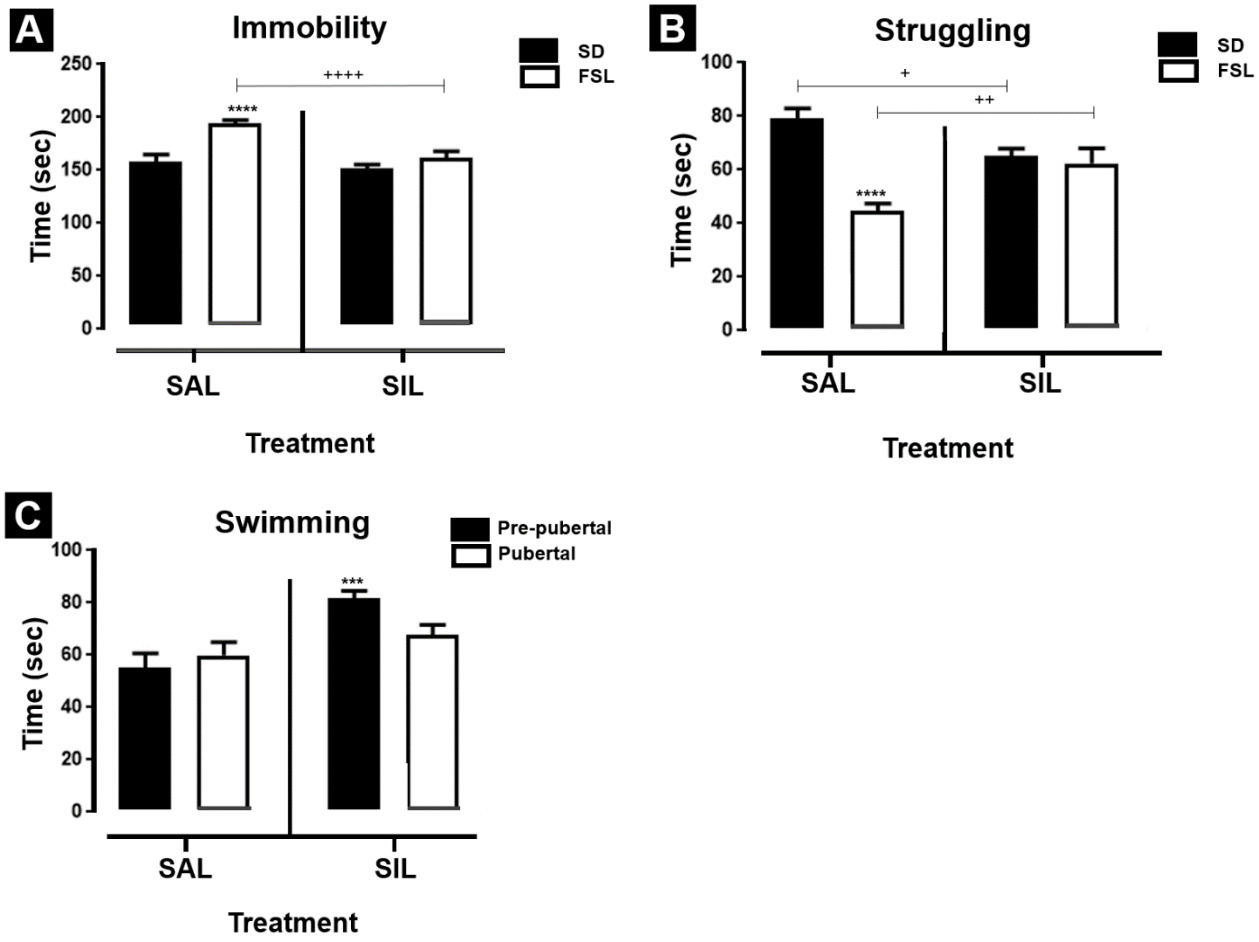

Figure 3-3: Effects of sub-chronic pre-pubertal (PnD 21–34) or pubertal (PnD 35–48) vehicle control or sildenafil treatment on depressive-like behaviour of SD and FSL rats in early adulthood (PnD 60). (A) Time spent immobile in the FST on PnD 60 following treatment of SAL+SD (n = 23), SAL+FSL (n = 24), SIL+SD (n = 24) or SIL+FSL (n = 24). (B) Time spent struggling in the FST on PnD 60 following treatment of SAL+SD (n = 23), SAL+FSL (n = 23), SIL+SD (n = 23) or SIL+FSL (n = 24). (C) Time spent swimming in the FST on PnD 60 following treatment of SAL+pre-pubertal (n = 23), SAL+pubertal (n = 24), SIL+pre-pubertal (n = 24) or SIL+pubertal (n = 24). Data points represent the mean ± SEM. Statistical analyses are reported in the text with *** $p \leq 0.001$, **** $p \leq 0.0001$ vs. SAL+SD for (A) and (B) or SAL+pre-pubertal for (C); ++ $p \leq 0.01$, +++ $p \leq 0.001$, ++++ $p \leq 0.0001$ vs. indicated test group. With abbreviations: SAL = saline, SIL = sildenafil, FSL = Flinders Sensitive Line rats and SD = Sprague-Dawley rats.

In Figure 3-3(A), there was no statistically significant three-way interaction between treatment, age and strain ($F_{1.88} = 1.305$, $p = 0.2564$, $\eta^2 = 0.015$) for time spent immobile in the FST on PnD 60. However, there was a statistically significant two-way interaction between treatment and strain ($F_{1.88} = 6.804$, $p = 0.011$, $\eta^2 = 0.072$). Consequently, Tukey's post-hoc test indicated that SAL-treated FSL rats were 36.30 sec (95% CI 17.70 to 54.91 sec) more immobile in the FST, compared to SAL-treated SD rats, irrespective of age ($p < 0.0001$). Moreover, SIL-treatment reduced time spent immobile in FSL rats by 32.84 sec (95% CI 14.24 to 51.45 sec), compared to SAL-treated FSL controls, irrespective of age ($p < 0.0001$). Still, SIL-treated SD rats spent

42.61 sec (95% CI 24.00 to 61.21 sec) less immobile, in relation to SAL-treated FSL rats, irrespective of age ($p < 0.0001$).

In Figure 3-3(B), there was no statistically significant three-way interaction between treatment, age and strain ($F_{1.88} = 0.006$, $p = 0.937$, $\eta^2 \leq 0.0005$) for time spent struggling in the FST on PnD 60. However, there were statistically significant simple two-way interactions between treatment and strain ($F_{1.88} = 19.4$, $p < 0.0001$, $\eta^2 = 0.181$), and strain and age ($F_{1.88} = 4.891$, $p = 0.030$, $\eta^2 = 0.053$), respectively ($F_{1.88} = 4.891$, $p = 0.0296$, $\eta^2 = 0.053$), with the former accepted as being the most robust (see discussion in statistical analyses section). Consequently, the Tukey's post-hoc test indicated that SAL-treated FSL rats spent 34.91 sec (95% CI 20.95 to 48.87 sec) less struggling in the FST (95% CI 20.05 to 49.05 sec), compared to SAL-treated SD rats, irrespective of age ($p < 0.0001$). SIL-treatment increased struggling behaviour by 17.86 sec (95% CI 3.90 to 31.83 sec) in FSL rats ($p = 0.006$) yet reduced it by 14.45 sec (95% CI 0.49 to 28.41 sec) in SD rats ($p = 0.004$), compared to their respective SAL-treated controls, irrespective of age. However, SIL-treated FSL rats displayed significantly less time struggling, compared to SAL-treated SD rats, irrespective of age ($p = 0.01$), as did SAL-treated FSL rats, compared to SIL-treated SD rats, irrespective of age ($p = 0.001$).

In Figure 3-3(C), there was no statistically significant three-way interaction between treatment, age and strain ($F_{1.88} = 1.791$, $p = 0.184$, $\eta^2 = 0.020$) for time spent swimming in the FST on PnD 60. However, there was a statistically significant two-way interaction between treatment and age ($F_{1.88} = 4.735$, $p = 0.032$, $\eta^2 = 0.051$). Consequently, Tukey's post-hoc test indicated that SIL increased the time spent swimming in the FST in pre-pubertal rats by 26.48 sec (95% CI 10.58 to 42.44 sec), compared to SAL-treated age-matched controls, irrespective of strain ($p = 0.0002$). Furthermore, animals treated with SIL during pre-pubertal development, also spent 21.78 sec (95% CI 5.85 to 37.71 sec) more time swimming in relation to animals treated with SAL during pubertal development, irrespective of strain ($p = 0.003$).

3.4.3 Anxiety-like behaviour

Figure 3-4 below depicts the later-in-life effects of early-life vehicle control or sildenafil treatment on the anxiety-like behaviour of SD and FSL rats on PnD 60.

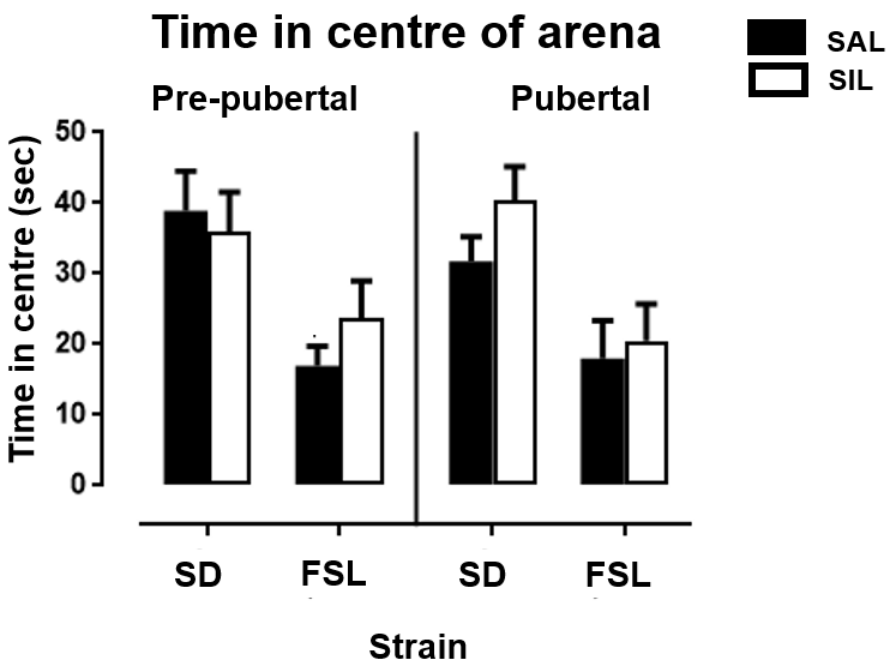

Figure 3-4: Effects of sub-chronic pre-pubertal (PnD 21–34) or pubertal (PnD 35–48) vehicle control or sildenafil treatment on the anxiety-like behaviour of SD and FSL rats in early adulthood (PnD 60). Time spent in the centre zone of the OFT on PnD 60 following treatment of SAL+SD (n = 24), SAL+FSL (n = 24), SIL+SD (n = 24) or SIL+FSL (24). Data points represent the mean ± SEM. With abbreviations: SAL = saline, SIL = sildenafil, FSL = Flinders Sensitive Line rats and SD = Sprague-Dawley rats.

In Figure 3-4, there was no statistically significant three-way interaction between treatment, age and strain ($F_{1.88}$ = 1.313, p = 0.255, η^2 = 0.015) for time spent in the centre zone of the OFT, nor any significant two-way interactions.

3.4.4 Cognitive function

Figure 3-5 below depicts the later-in-life effects of early-life vehicle control or sildenafil treatment on cognitive function in SD and FSL rats.

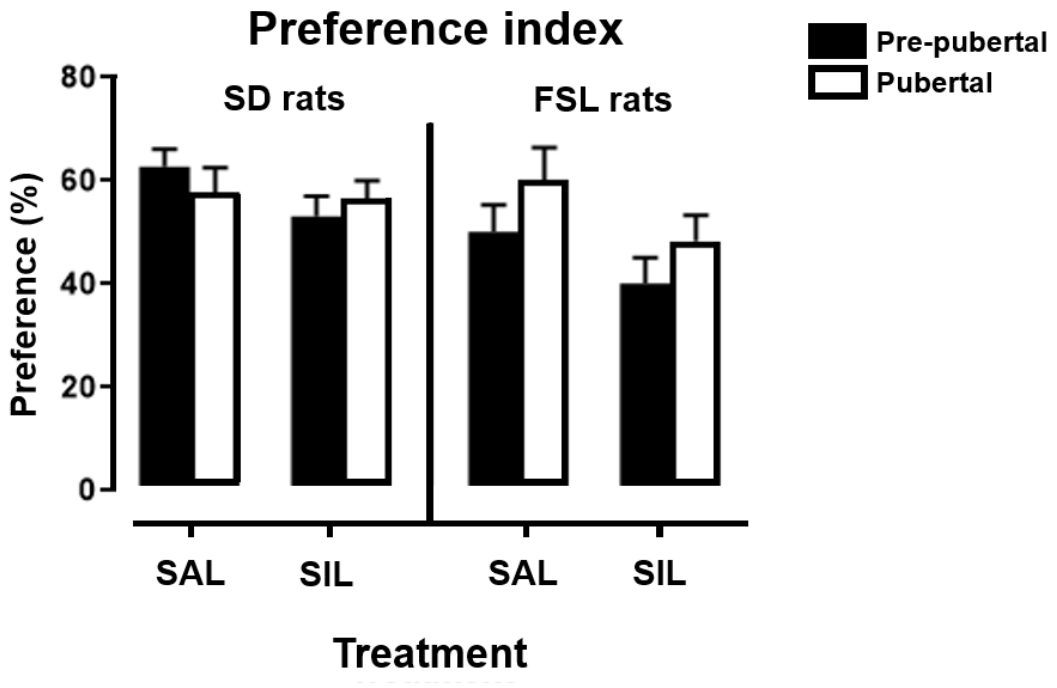

Figure 3-5: Effects of sub-chronic pre-pubertal (PnD 21–34) or pubertal (PnD 35–48) vehicle control or sildenafil treatment on the cognition of SD and FSL rats in early adulthood (PnD 60). Graphical representation of preference for the novel object (i.e. values > 50%), familiar object (i.e. values < 50%) or no preference between the novel and familiar objects (50%) in the nORT that was conducted on PnD 60 following treatment of SAL+SD+pre-pubertal (n = 12), SAL+SD+pubertal (n = 12), SIL+SD+pre-pubertal (n = 12), SIL+SD+pubertal (n = 12), SAL+FSL+pre-pubertal (n = 12), SAL+FSL+pubertal (n = 12), SIL+FSL+pre-pubertal (n = 12), SIL+FSL+pubertal (n = 12). Data points represent the mean ± SEM. With abbreviations: SAL = saline, SIL = sildenafil, FSL = Flinders Sensitive Line rats and SD = Sprague-Dawley rats.

In Figure 3-5, there was no statistically significant three-way interaction between treatment, age and strain ($F_{1.88}$ = 0.5927, p = 0.4435, η^2 = 0.007) for the preference index in the nORT on PnD 60, nor any statistically significant two-way interactions.

3.5 Discussion

The current study investigated the (1) later-in-life effects of pre-pubertal or pubertal SIL treatment on depressive- and anxiety-like behaviour and cognition in adulthood, (2) role of genetic susceptibility in the manifestation of such later-in-life effects, by comparing treatment outcomes of FSL rats (a genetic animal model of MDD) to that of control Sprague-Dawley rats, and (3) age at which sildenafil treatment could potentially induce the most robust later-in-life antidepressant-, anxiolytic-like and pro-cognitive effects, i.e. during pre-puberty, puberty or both.

3.5.1 Locomotor activity

Pre-pubertal and pubertal SIL treatment had no significant effect on general locomotor activity of either young adult SD or FSL rats (see Figure 3-2). This finding is in line with previous studies conducted in our laboratories showing that sub-chronic SIL treatment had no effect on the mean locomotor activity of male adult FSL rats (Brink *et al*., 2008; Liebenberg *et al*., 2010; Liebenberg *et al*., 2012). This is of note, since it implies that any changes in physical activity levels in the forced swim test and the open field test, would result from changes in psychomotor as opposed to locomotor activity, thereby rendering the data from these behavioural tests appropriate for delineating depressive- and anxiety-like effects.

3.5.2 Depressive-like behaviour

As expected, SAL-treated FSL rats displayed with a significantly increased depressive-like behaviour (i.e. increased immobility in the FST), as compared to SAL-treated SD rats in adulthood, regardless of age of juvenile treatment, confirming the depressive-like phenotype of FSL rats in our experimental setting (Porsolt *et al*., 1977; Overstreet, 1993; Overstreet *et al*., 1995; Lucki, 1997; Yadid *et al*., 2000; Friedman *et al*., 2007). This was seen regardless the juvenile age (i.e. pre-pubertal or pubertal) of treatment. The most important observation of this study is that both pre-pubertal and pubertal SIL treatment significantly reduced the depressive-like behaviour (i.e. immobility in the FST; Figure 3-3(A)) in adult FSL rats. Considering that the serotonergic system has matured in both these age groups as a common factor (compared to the noradrenergic or dopaminergic systems matured only in pubertal animals), serotonergic mechanisms may putatively underlie the SIL-induced antidepressant-like effects later in life. This was not, however, seen in control SD rats, suggesting the role of genetic susceptibility (as in FSL rats) for this effect to manifest.

Several previous studies from our and other laboratories showed that sub-chronic SIL treatment of adult FSL rats results in reduced depressive-like behaviour (Brink *et al*., 2008; Liebenberg *et al*., 2010; Baek *et al*., 2011; Liebenberg *et al*., 2012; Matsushita *et al*., 2012; Tomaz *et al*., 2014; Wang *et al*., 2014; Socala *et al*., 2016). However, it is important to note that these studies investigated the antidepressant-like effects of SIL immediately following sub-chronic treatment during adulthood, whereas the current study investigated the antidepressant-like effects of SIL later in life following a “wash-out” period. Importantly, as mentioned above, since no SIL-induced changes in locomotor activity was observed in the OFT (above), all changes in immobility in the FST can be interpreted as changes in psychomotor activity, so that reduced immobility can indeed be interpreted as antidepressant-like effects.

FSL rats seem to have impaired noradrenergic neurotransmission relative to SD rats, as can be seen from SAL-treated FSL rats that displayed significantly decreased struggling behaviour (Detke *et al*., 1995; Cryan *et al*., 2002; Slattery & Cryan, 2012), compared to SAL-treated SD rats in adulthood, regardless of age of treatment (compare Figure 3-3(B)). In addition, SIL seemed to reduce noradrenergic neurotransmission in SD rats, as can be seen from SIL-treated SD rats that displayed significantly reduced struggling behaviour compared to SAL-treated SD rats in adulthood, regardless of age of treatment (also compare Figure 3-3(B)). Therefore, it cannot be ruled out that SIL treatment of juveniles may have detrimental effects in normal healthy individuals in terms of hampering otherwise normal coping responses. Opposite from what was observed in SD rats, sildenafil seemed to increase noradrenergic neurotransmission in FSL rats, as can be seen from SIL-treated FSL rats that displayed a significantly increased struggling behaviour compared to SAL-treated FSL rats in adulthood, regardless of treatment age. These data would suggest that genetic susceptibility also plays a significant role in how later-in-life effects of juvenile SIL treatment on noradrenergic neurotransmission manifest in adulthood, particularly that in genetically susceptible animals SIL has later-in-life pro-adrenergic effects.

In addition, SIL seems to have increased serotonergic neurotransmission in rats in an age-dependent fashion, as can be seen from SIL-treated pre-pubertal, but not pubertal, rats that displayed significantly increased swimming behaviour (Detke *et al*., 1995; Cryan *et al*., 2002; Slattery & Cryan, 2012) compared to SAL-treated pre-pubertal rats in adulthood (compare Figure 3-3(C)), regardless of genetic susceptibility (i.e. in both SD and FSL rats). Our data suggest that enhanced monoaminergic signalling, i.e. enhanced serotonergic signalling in FSL rats, is implicated in the delayed (later-in-life) antidepressant action of SIL following early-life treatment. Since the serotonergic and noradrenergic pathways are still maturing during predominantly the pre-pubertal, and less so during the pubertal period (Murrin et al., 2007), these results suggest that early-life SIL treatment may have beneficial neurodevelopmental effects.

3.5.3 Anxiety-like behaviour

Neither pre-pubertal nor pubertal SIL treatment induced any significant alterations in anxiety-like behaviour in either the SD or FSL rats on PnD 60 (see Figure 3-4). This is of note since a previous study in our laboratories demonstrated sub-chronic SIL treatment to induce anxiolytic-like effects in adult FSL rats (Liebenberg *et al*., 2012). However, apart from the different treatment ages of the aforementioned and current studies, the behavioural tests also differed. In fact, Liebenberg and colleagues employed the social interaction test, which could be considered as a measurement of anxiety-like behaviour in a social context, which may be

different from anxiety-like behaviour in the OFT that is related to insecurity in an open environment whilst being alone. Since the same arena was used for the nORT analysis and the subsequent OFT in the current study, the animals may also already have acclimatized to the test arena, resulting in decreased exploratory behaviour, and thereby influencing anxiety-like behaviour.

Furthermore, several currently approved antidepressants have antidepressant action at lower doses than that needed for anxiolytic action (Cassano *et al*., 2002) and the lack of anxiolytic effects in this study may largely be dose-dependent. We therefore suggest that anxiety-like behaviour be further investigated in the social interaction test, or in other, more robust behavioural models, such as the elevated plus maze. Moreover, there were also no significant differences between the anxiety-like behaviour of SAL-treated SD, and SAL-treated FSL rats, regardless of treatment age. This is in line with the reported absence of elevated anxiety-like behaviour observed in the FSL rats as compared to control animals, despite their enhanced sensitivity to stress (Overstreet *et al*., 2005; Neumann *et al*., 2011).

3.5.4 Cognition

Pre-pubertal and pubertal SIL treatment had no significant effect on the mean cognitive function of SD or FSL rats in adulthood (see Figure 3-5). These findings are in line with previous reports of an insignificant effect of select antidepressant treatments on MDD-induced cognitive deficits. (Pacher & Kecskemeti, 2004; Rosenzweig-Lipson *et al*., 2007). Yet, studies have reported PDE5 inhibitors (such as SIL) to ameliorate cognitive function via a reduction in β-amyloid-induced neuroinflammation in animal models of Alzheimer's disease (Puzzo *et al.*, 2009; Palmeri *et al.*, 2013; Zhang *et al.*, 2013; Zhang *et al.*, 2018) and several other preclinical studies have also demonstrated procognitive effects of SIL treatment (Devan *et al.*, 2004; Rutten *et al.*, 2008a; Reneerkens *et al.*, 2009; Boccia *et al.*, 2011).

One study also investigated the procognitive effects of SIL treatment in healthy young and aged rodents and found that sub-chronic SIL treatment had greater procognitive effects in aged rodents compared to young rodents (Puzzo *et al.*, 2014) and findings from this study strongly suggest that the inhibition of PDE5 can counteract apoptosis during aging by modulating pro- and antiapoptotic molecules (Puzzo *et al.*, 2014). As such, the procognitive effects of SIL treatment appear to be in part dependent on treatment age (Puzzo *et al.*, 2014) and this may explain the lack of later-in-life procognitive effects of juvenile SIL treatment in the current study. Furthermore, indecisiveness is an important clinical diagnostic criterion for MDD (American Psychiatric Association, 2013) and may also explain the lack of preference displayed by the rats for either the familiar or novel object in the nORT. In the current study there were also no

significant differences between the cognitive functioning of SAL-treated SD and SAL-treated FSL rats in adulthood, irrespective of age of treatment.

3.6 Conclusion

Early-life (i.e. pre-pubertal and pubertal) sildenafil treatment has favourable effects on the depressive-like behaviour of stress-sensitive FSL rats in adulthood. In addition, genetic susceptibility plays a significant role in this effect of sildenafil later in life, since it was not observed in adult SD rats (i.e. rats without a genetic predisposition to develop MDD). There is also evidence that sildenafil may promote later-in-life pro-noradrenergic effects in stress-sensitive FSL rats, and that sildenafil has age-dependent pro-serotonergic effects. However, these favourable effects of early-life sildenafil treatment in FSL rats do not apply for anxiety-like behaviour or the improvement of cognitive functioning in adulthood. As a working hypothesis, when translating the current results to humans, it may be that patients with a genetic predisposition to develop MDD may benefit from early-life sildenafil treatment, putatively by modulating neurodevelopment and thereby to result in significantly decreased susceptibility to develop MDD in adulthood. This promising idea warrants further investigation. In addition, future pre-clinical studies, investigating the neurochemical effects of pre-pubertal and pubertal sildenafil treatment, as well as the immediate bio-behavioural effects of early-life sildenafil treatment, may better our understanding of the long-term safety, efficacy and adverse effects of sildenafil treatment during early-life development.

3.7 Compliance with Ethical Standards

The current study, along with all of the animal procedures, were approved by the NWU-AnimCare Animal Research Ethics Committee (NHREC reg. no. AREC-130913-015) of the North-West University (*approval number:* ***NWU-00277-17-S5***). All experiments adhered to the guidelines of the South African National Standards: The care and use of animals for scientific purposes (SANS 10386:2008). Furthermore, all animals were maintained, and all procedures performed in studies involving animals were in accordance with the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation.

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3.9 Conflict of interest

Except for research funding granted to Prof Christiaan B Brink from the NRF, no financial support or compensation was received from any corporate entity or individual over the past two years for research or professional services. There are also no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Chapter 4. Summary, discussion, conclusion and suggestions for future studies

Chapter 4 provides a comprehensive summary of the results obtained during this study in order to come to an appropriate conclusion and to formulate recommendations for prospective studies. The experimental results discussed in this chapter represent the results obtained from the behavioural assays as conducted on postnatal day (PnD) 60, following pre-pubertal or pubertal sildenafil treatment (see *Chapter 3*), and the results from the brain-derived neurotrophic factor (BDNF) analyses, not included in the main findings (see *Addendum B*). The methods for the preparation and performing of the behavioural assays employed in this study can be found in *Chapter 3*, whereas background information and more detail on these behavioural assays can be found in *Addendum A*. The methods for the preparation and performing of BDNF analyses are discussed in *Addendum A*, whereas the results of the BDNF analyses are discussed more broadly in *Addendum B*.

Aims and objectives of this study include (see section 1.3):

- To investigate in a translational genetic animal model of depression (i.e. the FSL rat) whether pre-pubertal and/or pubertal sub-chronic sildenafil administration, versus vehicle-control (i.e. saline), induces any later-in-life bio-behavioural effects, as displayed in adulthood (following a “wash-out” period), including modulation of depressive- and anxiety-like behaviour, cognition and neurobiological markers of depression;
- To investigate the role of genetic susceptibility in any of the observed later-in-life bio-behavioural effects of early-life sildenafil treatment, by comparing results obtained from FSL rats (i.e. a genetic animal model of depression) to that obtained from control SD rats; and
- To investigate the role of juvenile age of sildenafil treatment in any bio-behavioural effects observed in adulthood, by comparing results obtained from pre-pubertal-treated to that of pubertal-treated rats.

4.1 Summary of results

The key findings of this study include:

- Flinders Sensitive Line (FSL) rats present with significantly increased immobility in the forced swim test (FST) (i.e. depressive-like behaviour) compared to a behavioural control, i.e. Sprague-Dawley (SD) rats.
- FSL rats present with significantly decreased struggling behaviour in the FST (i.e. noradrenergic-associated behaviour) compared to a behavioural control (i.e. SD rats).
- Early-life (i.e. pre-pubertal and pubertal) sub-chronic sildenafil treatment reduces immobility in the FST (i.e. depressive-like behaviour) in adult FSL rats but not in SD rats, as observed in the FST.
- Early-life (i.e. pre-pubertal and pubertal) sub-chronic sildenafil treatment increases struggling behaviour (i.e. noradrenergic-associated behaviour) in adult FSL rats and decreases struggling behaviour in adult SD rats, as observed in the FST.
- Pre-pubertal sub-chronic sildenafil treatment increases swimming behaviour in the FST (i.e. serotonergic-associated behaviour) in both FSL and SD rats in adulthood.
- Early-life (i.e. pre-pubertal and pubertal) sub-chronic sildenafil treatment does not affect the time spent in the centre of the open field test (OFT) (i.e. a measure of anxiety-like behaviour) in adulthood.
- Early-life (i.e. pre-pubertal and pubertal) sub-chronic sildenafil treatment does not affect the time spent exploring the novel object in the novel object recognition test (nORT) (i.e. cognition in terms of memory) in adulthood.
- Early-life (i.e. pre-pubertal and pubertal) sildenafil treatment does not have a significant effect on BDNF concentrations in the hippocampi of adult rats (i.e. both FSL and SD).

4.2 Discussion and conclusion

Early-life (i.e. pre-pubertal and pubertal) saline-treated FSL rats displayed with significant innately enhanced depressive-like behaviour (i.e. increased immobility in the FST) compared to saline-treated SD rats in adulthood, confirming the face validity of the FSL rat model of MDD under our experimental conditions in the current study. This finding was expected, since depressive-like behaviour, including in the FST, is well documented for the FSL rat as a validated genetic animal model of MDD (see section 2.9.2) (Porsolt *et al.*, 1977b; Overstreet, 1993; Overstreet *et al.*, 1995; Lucki, 1997; Yadid *et al.*, 2000; Friedman *et al.*, 2007). In fact, early-life (i.e. pre-pubertal and pubertal) sub-chronic sildenafil treatment significantly reduced depressive-like behaviour in FSL rats (with a genetic susceptibility to develop MDD), but not in

SD rats (without a genetic susceptibility to develop MDD) in adulthood. This observation suggests that the later-in-life antidepressant-like effects of early-life sildenafil exposure are dependent on genetic susceptibility to develop MDD. This further implies that sildenafil will only display its antidepressant-like effect (here specifically referring to enhanced immobility) in depressive-like animals. This is in line with clinical findings showing that antidepressants in general do not have euphoric effects in healthy individuals but display mood elevating effects in depressed patients.

A previous study conducted in our laboratories demonstrated antidepressant-like effects directly after sub-chronic sildenafil treatment in adult FSL rats at the same dosage as was implemented in the current study (i.e. 3 mg/kg/day) (Liebenberg *et al.*, 2010a). The antidepressant-like effects of sildenafil have subsequently been demonstrated by various other studies (Baek *et al.*, 2011a; Matsushita *et al.*, 2012; Tomaz *et al.*, 2014; Wang *et al.*, 2014a; Socała *et al.*, 2016). The current study thus demonstrated that sub-chronic sildenafil treatment not only has immediate effects, but also later-in-life, antidepressant-like effects following early-life treatment. The difference in duration of the "wash-out" periods between pre-pubertal- and pubertal-treated rats could be an important consideration when interpreting the findings from this study, however our data suggest that the difference in duration of the "wash-out" periods had no significant effect, except for potential effects on the swimming behaviour of rats. Importantly, since no sildenafil-induced alterations in general locomotor activity were seen in the OFT, all alterations in immobility in the FST can be interpreted as alterations in psychomotor activity, so that reduced immobility can indeed be interpreted as antidepressant-like effects. Since the FSL rat has been shown to possess robust predictive validity, this is a valuable indication that it will translate to the human condition, warranting further investigation.

Moreover, early-life saline-treated FSL rats displayed with significantly reduced noradrenergic-associated behaviour (i.e. struggling behaviour) (Detke *et al.*, 1995; Cryan *et al.*, 2002; Slattery & Cryan, 2012) compared to saline-treated SD rats in adulthood in the current study. In this regard, FSL rats have been shown before to display decreased struggling behaviour in the FST compared to behavioural controls (Oberholzer *et al.*, 2018). In the current study, early-life sub-chronic sildenafil treatment significantly increased noradrenergic-associated behaviour in adult FSL rats, whereas early-life sub-chronic sildenafil treatment significantly reduced noradrenergic-associated behaviour in adult SD rats. The latter suggests that early-life sildenafil treatment may have detrimental effects in normal, healthy rats in terms of impairing otherwise normal coping responses. The effects of sildenafil are therefore not limited to the FSL rats. This finding further highlights the significant role that genetic susceptibility to develop MDD plays in the effects of early-life sub-chronic sildenafil treatment. Interestingly, pre-pubertal sub-chronic sildenafil treatment increased serotonergic-associated behaviour (i.e. swimming) (Detke *et al.*,

1995; Cryan *et al.*, 2002; Slattery & Cryan, 2012) in both FSL and SD rats in adulthood. However, pubertal sub-chronic sildenafil treatment had no significant effect on serotonergic-associated behaviour in either FSL or SD rats in adulthood.

Importantly, the serotonergic system reaches maturity during pre-puberty, whereas the noradrenergic system continues developing throughout pre-pubertal development, only reaching maturity during puberty (Murrin *et al*., 2007). In this regard, potential effects on the serotonergic system can manifest following pre-pubertal and pubertal sildenafil treatment, whereas potential effects on the noradrenergic system only manifests following pubertal sildenafil treatment. That said, pre-pubertal and pubertal sildenafil treatment in this study display antidepressant-like effects in adult FSL rats (i.e. reduced immobility in the FST), whereas pre-pubertal and pubertal sildenafil treatment display no significant effect on the immobility of adult SD rats in the FST. Since sildenafil reduced immobility in both pre-pubertal- and pubertal-treated FSL rats and considering that only the serotonergic system has matured in both these age groups, it is likely that sildenafil brought the later-in-life antidepressant-like effects about through an unknown serotonergic mechanism. The differences in noradrenergic-associated behaviour observed between FSL and SD rats in adulthood further suggest that genetic susceptibility to develop MDD plays a role in sildenafil's antidepressant-like effects observed in the FST.

There was no significant difference in anxiety-like behaviour between early-life saline-treated FSL and SD rats. This finding is in line with the reported absence of elevated anxiety-like behaviour observed in FSL rats, despite their enhanced sensitivity to stress (Overstreet *et al.*, 2005; Neumann *et al.*, 2011). Early-life sub-chronic sildenafil treatment had no effect on anxiety-like behaviour in both adult FSL and SD rats, indicating that genetic susceptibility to develop MDD has no influence on sildenafil's effects (or the lack thereof) on anxiety-like behaviour. However, the data also suggest that early-life sub-chronic sildenafil treatment does not enhance anxiety-like behaviour in adulthood, as has been suggested for juvenile antidepressant treatment.

Furthermore, there was no significant difference in cognition between saline treated FSL and SD rats in adulthood in the current study. Also, early-life sub-chronic sildenafil treatment had no effect on cognition in FSL and SD rats in adulthood. A possible reason for this observation is that sildenafil's procognitive effects have been shown to be more pronounced in adult compared to juvenile rats (Puzzo *et al.*, 2014). Both saline-treated FSL and SD rats had comparable BDNF levels in adulthood (data shown in *Addendum B*). Sub-chronic sildenafil treatment did not significantly affect BDNF concentrations (i.e. a marker of neuroplasticity) in either adult FSL or SD rats. Since enhanced BDNF levels has been shown to be associated with enhanced

object recognition memory in the nORT (Bechara & Kelly, 2013), this finding may explain the lack of effects of sildenafil on cognition.

Nevertheless, it cannot be excluded that sildenafil initially could have increased BDNF during early-life treatment, which may have affected neurodevelopment, and then may have manifested in adulthood, after lowering of BDNF levels, as antidepressant-like effects and effects on noradrenergic and serotonergic neurotransmission, as discussed above. Pre-pubertal and pubertal sub-chronic sildenafil treatment induced similar bio-behavioural and cognitive effects in adult FSL and SD rats, with only swimming behaviour as an exception. In this instance, only pre-pubertal sub-chronic sildenafil treatment increased swimming behaviour in both FSL and SD rats, whereas pubertal sub-chronic sildenafil treatment had no significant effect on the swimming behaviour of adult FSL and SD rats. Finally, welfare monitoring of animals (results not discussed elsewhere) suggest that early-life sub-chronic sildenafil treatment has no observable adverse effects in FSL rats into adulthood.

In conclusion, early-life sub-chronic sildenafil treatment (i.e. pre-pubertal or pubertal) induces later-in-life antidepressant-like effects in adult FSL rats (i.e. rats with a genetic susceptibility to develop MDD), without any significant antidepressant-like effects on adult SD rats (i.e. rats without a genetic susceptibility to develop MDD). Therefore, genetic susceptibility to develop MDD appears to play a significant role in sildenafil's antidepressant action. From the current study it appears as if the antidepressant-like activity of sildenafil may in part be through a noradrenergic mechanism (only rats with increased struggling behaviour following sub-chronic sildenafil treatment displayed antidepressive-like effects), but neurochemical studies are warranted. Early-life sub-chronic sildenafil treatment had no effect on anxiety-like behaviour, general locomotor activity or cognition of adult FSL and SD rats. BDNF concentrations in the hippocampi of adult FSL and SD rats were not significantly altered by early-life sub-chronic sildenafil treatment.

This study suggests that early-life sub-chronic sildenafil treatment has later-in-life antidepressant-like effects in rats genetically predisposed to develop MDD. Future pre-clinical studies investigating the later-in-life neurochemical effects and the immediate bio-behavioural effects of early-life (pre-pubertal and pubertal) sildenafil treatment may better our understanding of the long-term safety and efficacy of sildenafil treatment during early-life development. As a working hypothesis, when translating these results to humans, patients with a genetic predisposition to develop MDD may benefit from the later-in-life antidepressive effects of early-life sub-chronic sildenafil treatment. This warrants further investigation.

4.3 Suggestions for future studies

The current study did achieve all of the aims and objectives outlined in section 1.3, but there were several limitations to the current study. Regarding these limitations, recommendations for prospective studies include:

- The current study made use of a dose of 3 mg/kg/day of sildenafil, which was calculated from an effective dose of sildenafil in adult rats. With that said, a dose-response study in young rats may be valuable to identify the optimal dose(s) of sildenafil in young (i.e. pre-pubertal and pubertal) rats for future studies.
- The current study only interpreted behavioural data with hippocampal BDNF data. Brain tissue is available to correlate behavioural data with more biomarkers in future studies and it may be valuable to examine in the prefrontal cortex the effects of early-life sildenafil treatment on monoamines and their metabolites, the expression of cGMP, the expression of PDE5, monoaminergic transporters, and NO metabolite and NOS concentrations in adulthood. Studying these neurochemical biomarkers will assist in uncovering the neurobiological mechanisms by which later-in-life antidepressive-like effects are induced into adulthood following early-life sildenafil treatment.
- The current study did not investigate the immediate effects or the effects beyond 60 days of pre-pubertal and pubertal sildenafil exposure. Therefore, it is not known whether sildenafil-induced alterations occur immediately after exposure and if the antidepressive-like effects persist into old age. It may be valuable to investigate these possibilities.
- This study only investigated the effects of one PDE5 inhibitor, i.e. sildenafil. Future studies may compare the bio-behavioural effects of different PDE5 inhibitors, e.g. tadalafil. This would give us insights into whether the bio-behavioural effects observed in this study are exclusive to sildenafil or do these effects extend to other PDE5 inhibitors.
- Future studies may consider measuring BDNF concentrations immediately after sub-chronic sildenafil treatment to establish whether there is an immediate increase in BDNF concentrations after sildenafil treatment that eventually return to baseline concentrations in later life.
- Following early-life sub-chronic sildenafil treatment in FSL and SD rats, a stressor can be introduced, and the later-in-life bio-behavioural and cognitive effects can subsequently be evaluated (i.e. assessing sildenafil-induced improvements in stress resilience).
- The irritability/aggression of young pre-treated and adult rats may be assessed through behavioural analyses and the irritability/aggression of young FSL rats may be compared

to that of young SD and/or adult FSL rats, since irritability/aggression frequently occurs in depressed children.

- A non-injected control group may also be included in the study design to shed light on the possible effects of injection stress on the results obtained.
- Future studies may benefit from conducting the OFT prior to the nORT when using the same arena for both tests. In assessments of inherent, novel place anxiety (i.e. during the OFT), rats should not be familiar with the testing arena used.
- A saline-treated FSL and SD group for neurochemical analyses that have not been subjected to the battery of behavioural tests prior to euthanization may be included in future studies to determine if the battery of behavioural tests affect the results obtained during neurochemical analyses.
- Finally, the manner in which sildenafil may influence serotonergic neurotransmission needs further investigation. For instance, measuring specific biomarker concentrations (e.g. monoamines, cGMP and NO metabolites), serotonergic transporter expression and serotonergic receptor densities (e.g. 5-HT_{1A}).

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Addendum A: Materials and methods

Addendum A provides a discussion of all the materials and methods used in this study. Most of the materials and methods used in this study have already been discussed in Chapters 2 and 3 and in these instances reference to the appropriate sections is provided, with additional information where appropriate.

A.1 Animals

The current study made use of Flinders Sensitive Line (FSL) rats and a behavioural control, i.e. Sprague-Dawley (SD) rats, as discussed in sections 2.9.2 and 3.2.1.1.

A.1.1 General housing protocol

On PnD 21 rat pups were weaned and housed under standard laboratory conditions (see section 3.2.1.1) in cages of 2-3 rats per cage until PnD 60 when behavioural studies were conducted. During the treatment periods (i.e. PnD 21-34 and PnD 35-48) rats were housed under the same conditions.

A.1.2 Limiting the study to male rats only

The hormonal cycles of female rodents are known to influence behaviour, which may complicate the interpretation of data obtained from behavioural studies following interventions. Therefore, due to the hormonal cycles of female rats potentially influencing the results of the behavioural studies that were conducted in this study, only male rats were included. Although pre-pubertal female rats do not present with hormonal cycles as is observed in pubertal female rats (Murrin *et al.*, 2007), in order to accurately compare the results of the behavioural studies between pre-pubertal and pubertal treatment groups, only male pre-pubertal rats were included in this study. In addition, behavioural tests were conducted on PnD 60 and the hormonal cycles of female rats may affect their behaviour in behavioural tests and therefore the results of behavioural tests. For a broader discussion on limiting the study to male rats only see section 2.9.3.

A.2 Drug treatment

Sildenafil citrate was used in this study at a dose of 3 mg/kg/day, not requiring the co-administration of a muscarinic receptor antagonist (Liebenberg *et al.*, 2010a), administered through the subcutaneous (sc) route for 14 days. Rats received sildenafil treatment either during pre-puberty or puberty. See section 3.2.1.2 for a broader discussion on the drug used in this study.

Young rats are more vulnerable to injection injury compared to adult rats, especially with sub-chronic intraperitoneal administration. A previous study demonstrated that the bio-availability following a sc injection was only 2-3% lower compared to an intraperitoneal injection (Wright & Wilson, 1983). Therefore, sc and intraperitoneal injections can be considered to yield comparable drug concentrations, with sc injections having a decreased risk of causing injury. Accordingly, the sc injection route was used in this study.

A.3 Background and methods for the behavioural studies

The following section provides a discussion of each of the different behavioural tests that were conducted in this study. All behavioural tests were conducted according to the required guidelines and with prior ethical approval from the NWU-AnimCare Animal Research Ethics Committee (see section 1.7). In order to reduce the number of rats used in this study, rats were subjected to a battery of behavioural tests, arranged from least stressful to most stressful i.e. the novel object recognition test (nORT), open field test (OFT) and forced swim test (FST) (Mokoena *et al.*, 2015). All behavioural tests were conducted during the rat's dark cycle to ensure that the most accurate behavioural data were obtained from the rats. A brief discussion and history of the behavioural tests that were used in this study are provided below. Importantly, each behavioural test was separated by a habituation period of 1 hour to minimize the effects of stress, caused by moving the home cages from one room to another, on the behaviour of rats during the behavioural tests.

A.3.1 Novel object recognition test

The novel object recognition test (nORT) was first described in the late 1980's and has been used to assess memory performance in rats ever since (Ennaceur & Delacour, 1988). The nORT has a major advantage compared to the majority of behavioural tests, i.e. no aversive or stressful stimuli are required to conduct the nORT, and this may be the reason for its popularity and common use (Rutten *et al.*, 2008b). The nORT is based on the observation that rats prefer to explore a novel object above a familiar object (Rutten *et al.*, 2008b). The nORT has been

used in more than a thousand behavioural studies since it was developed in 1988 and different variations of the original test exist (Rutten *et al.*, 2008a).

The nORT comprised of three phases and was performed in a 1 m^2 square test arena, with opaque black, vertical walls (*h = 45 cm*). The nORT was conducted as previously described (Abildgaard *et al.*, 2011). **Figure A-1** below depicts the apparatus used for the nORT.

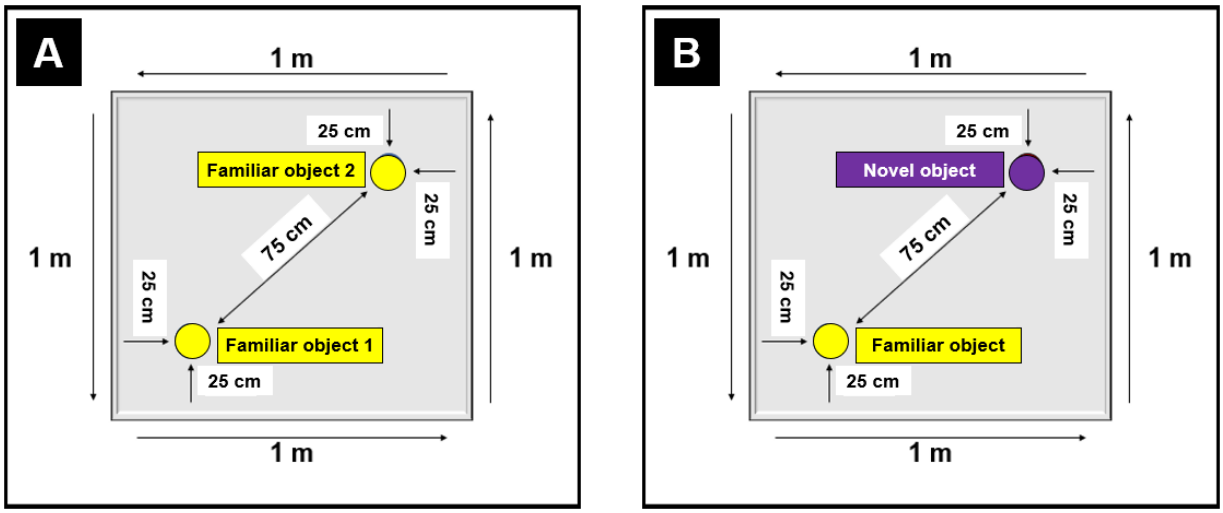

Figure A-1: An illustration of the apparatus used for the nORT, depicting the acquisition trial (A) and the retention trial (B).

In short, during the first phase, rats were placed in the centre of the OFT arena and allowed to explore the arena for 10 min. During the second phase (24 hours later), the rats were placed in the centre of the same arena, this time facing one of two identical objects that were placed in opposite corners of the arena (i.e. yellow plastic children's toy ducks), 25 cm from each wall, and allowed to explore the objects for 5 min (also known as the acquisition trial). During the final phase (90 min later), the rats were placed in the centre of the same arena, facing one of two objects that were placed in opposite corners of the arena, 25 cm from both walls, and allowed to explore the objects for 5 min (also known as the retention trial). However, this time one of the objects from the previous phase was replaced by a novel object (i.e. a purple owl glass salt pot). The objects were fastened to the floor of the arena to prevent the rats from moving the objects. Importantly, the arena and objects were wiped clean with a 10% ethanol solution between each trial to avoid any olfactory cues in subsequent trials. The exploratory behaviour of the rats during the nORT was video-recorded with a video camera installed directly above the OFT arena and the nORT was conducted under red light (80 lx).

A rat with unimpaired memory functioning will spend more time exploring the novel object relative to the known object in the third phase (indicative of memory consolidation). Object exploration was defined as the rat orienting itself towards the object and actively sniffing, licking

or physically touching the object (Abildgaard *et al.*, 2011). However, when rats were standing or sitting on the objects, they were not considered to be exploring the objects (Goff & Coyle, 2001). The video recordings were scored using Ethovision XT14 software (Noldus Information Technology BV, Wageningen, Netherlands).

The total time that the rats spent exploring the familiar object (T1), novel object (T2) and the familiar and novel objects combined (T1 + T2) in the final phase of the nORT was determined. Then, the preference index (PI) was calculated for each rat by using the following formula PI = $\frac{T2}{T1+T2}$ X 100 (Wang *et al*., 2007), where preference for the novel object is indicated by values greater than 50%, preference for the familiar object is indicated by values less than 50% and a value of 50% indicates no preference for the familiar or novel object (Hammond *et al.*, 2004).

A.3.2 Open field test

Since the open field test (OFT) was first developed, many variations of the test have been described and include arenas of different shapes (e.g. circular, square or rectangular), lighting differences (e.g. lighting from above or underneath the arena), the presence or absence of objects within the arena (e.g. platforms or tunnels), variations in the starting position of test subjects (e.g. in the centre or close to the walls of the arena) and different durations of the test (i.e. from 2 to 20 min, but usually 5 min). However, despite the differences in the setup of the OFT, the basis of the test remains the same, i.e. the animal is placed inside the open field arena and allowed to freely explore the arena for a predefined time. The OFT is one of the most popular and commonly used behavioural tests in animal psychology and the reason for this is the ability of the OFT to evaluate both anxiety-like behaviour and locomotor activity (Hall, 1934; Prut & Belzung, 2003).

With that said, the OFT was first described by Hall in 1934 and was initially developed to assess the emotional state of test subjects (Hall, 1934; Prut & Belzung, 2003). Interestingly, Hall assessed the behaviour of rats in a circular open field arena with vertical walls (1.2 m (*d*) x 0.45 m (*h*)) and noted that emotional rats tend to enter the central zone of an open field arena fewer times compared to controls (Hall, 1934). The time spent in the central zone of the open field arena is indicative of reduced anxiety-like behaviour (Prut & Belzung, 2003; Overstreet & Griebel, 2004) and the success of the OFT in identifying anxiety-like behaviour is built on two factors known to trigger anxiety-like behaviour in animals, i.e. individual testing (separation from a social group) and agoraphobia (fear of feeling trapped and/or helpless). Importantly, the behaviour of rodents in the OFT is mainly dependent on tactile sensory factors and studies have demonstrated that rodents without vibrissae (whiskers) display low levels of thigmotaxis (i.e. walking close to the walls of an open field arena), resulting in an elevated number of entries into

the central zone of an open field arena and could therefore be misinterpreted as anxiolytic-like behaviour. Moreover, food and water intake and lighting conditions may also affect the anxiety-like behaviour displayed by animals in the OFT (Walsh & Cummins, 1976; Prut & Belzung, 2003).

As previously mentioned, the OFT is also used to evaluate the general locomotor activity of animals. The general locomotor activity of animals is analysed in combination with their behaviour in the FST to support the possible depressive-like behaviour observed in the animals and to prevent false-negative results in the FST. It is pivotal to determine whether the general locomotor activity of an animal was influenced by an intervention and/or treatment, due to the results of the FST, as well as the interpretation thereof, being dependent on the time that the animal spent immobile in the FST. For example, if an animal presents with a significantly increased time spent immobile in the FST compared to controls following an intervention and/or treatment, but also presents with significantly reduced general locomotor activity in the OFT compared to controls, the physiological effect that the intervention and/or treatment has on the general locomotor activity of the animal may be misinterpreted as a psychological effect (Slattery & Cryan, 2012). However, due to the high comorbidity between anxiety disorders and MDD, it is suggested that MDD and anxiety disorders be investigated together and not in isolation from each other, making the OFT a valuable indicator of anxiety-like behaviour in MDD studies (Slattery & Cryan, 2012).

The OFT was conducted as previously described for our laboratories (Schoeman *et al.*, 2017; Steyn, 2018). The apparatus consisted of a square open field arena (1 m^2) with opaque black walls (h = 45 cm). On the day of testing, the rats were placed in the centre of the open field arena and allowed to explore the open field arena freely for 5 min under red light (80 lx). The behaviour of the rats during the OFT was recorded with a video-camera situated above the open field arena. The open field arena is depicted in **Figure A-2** below.

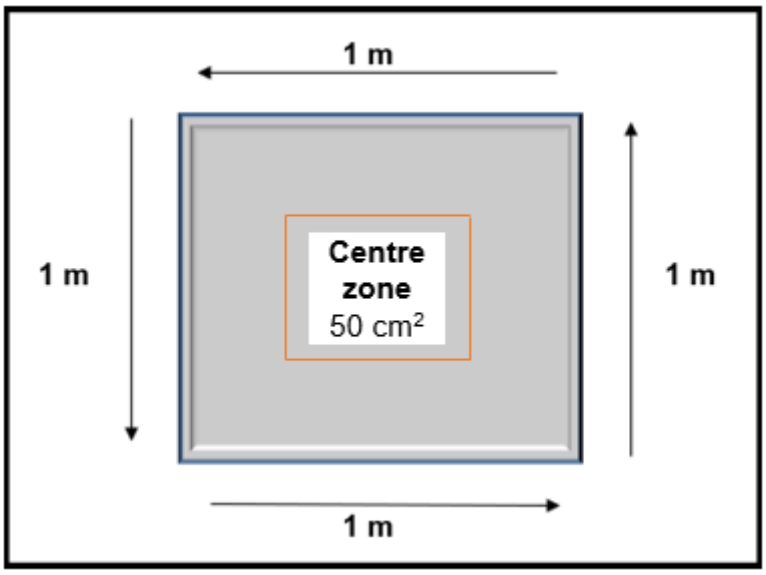

Figure A-2: An illustration of the apparatus used for the OFT.

Behaviour of the rats during the OFT was subsequently analysed using Ethovision XT14 software (Noldus Information Technology BV, Wageningen, Netherlands). Behavioural parameters that were measured during the OFT included the total distance covered by the rats (i.e. indicative of general locomotor activity) and the total time spent in the centre zone of the open field arena (i.e. indicative of reduced anxiety-like behaviour). This anxiety-like parameter has been shown to positively correlate with those measured by other anxiety-like behavioural tests, e.g. the elevated plus maze (Bergami *et al.*, 2009; Bhatia *et al.*, 2011).

A.3.3 Forced swim test

The forced swim test (FST) is used to assess antidepressant activity over a broad spectrum of antidepressants (Borsini & Meli, 1988) and is based on the observation that after rats have been placed into an inescapable cylinder filled with water, they will initially try and escape through escape-directed behaviour (Armario *et al.*, 1988). This escape-directed behaviour is then followed by rats developing an immobile posture which has been associated with a failure of perseverance in escape-directed behaviour, i.e. behavioural despair (Lucki, 1997; Petit-Demouliere *et al.*, 2005).

The FST was first described by Porsolt and colleagues (Porsolt *et al.*, 1977b), however a modified version of the FST has since been developed and is currently one of the most commonly used behavioural tests for depressive-like behaviour (Slattery & Cryan, 2012). The traditional FST comprises of two separate swim sessions. In this regard, healthy animals first undergo a 15 min pre-swim session and are then returned to their home cages. After 24 hours, the animals undergo a 5 min test swim session. The pre-swim session ensures that the animals

adopt an immobile posture more rapidly during the test session, which enables the effects of compounds being tested for antidepressant properties to easily be observed (Lucki, 1997; Cryan *et al.*, 2002). The behaviour of the animals during the test swim session is scored and subsequently analysed. When antidepressants are administered after the pre-swim session, animals persist in their escape-directed behaviour for a longer period of time during the subsequent test swim session compared to controls (Cryan *et al.*, 2002).

As previously mentioned, in the traditional FST, exposure to a 15 min pre-swim session is required 24 hours before the test swim session, however a pre-swim session is not required for the inherent elevated immobility of FSL rats to be detectable in the 5 min test swim session (Overstreet, 1993; Dremencov *et al.*, 2004; Overstreet & Griebel, 2004). Moreover, the elevated immobility of FSL rats in the FST is for the most part only reduced following sub-chronic or chronic (at least 14 days) antidepressant treatment, especially at low doses (Vazquez-Palacios *et al.*, 2004; Cryan *et al.*, 2005b; Overstreet & Wegener, 2013).

However, the traditional FST is unreliable in detecting antidepressant-like effects of SSRIs, although the SSRIs are known to be clinically effective in the treatment of MDD (Lucki, 1997). An adapted version of the FST was subsequently published in an effort to make the FST more sensitive to the antidepressant-like effects of SSRIs (Cryan *et al.*, 2002), whereby an increase in the water depth (from 30 cm to 40 cm) and the use of a time sampling technique in which the main behaviour of the rat during a 5 second time interval is scored (Cryan *et al.*, 2002). Another modification to the traditional FST protocol is that FSL rats can already be tested for antidepressive-like behaviour only 24 hours after the last antidepressant administration of a 14-day treatment regimen (Overstreet & Wegener, 2013), as implemented in the current study. These modifications enable investigators to distinguish between three types of behaviour during the FST:

- Swimming: horizontal movements within the cylinder (crossing from one quadrant of the swim cylinder into another);
- Struggling/climbing: upward-directed movements of the forepaws against the inside of the swim cylinder; and
- Immobility: only movements that are needed for the rat to keep its head above the water.

These three distinct behaviours are illustrated in **Figure A-3** below.

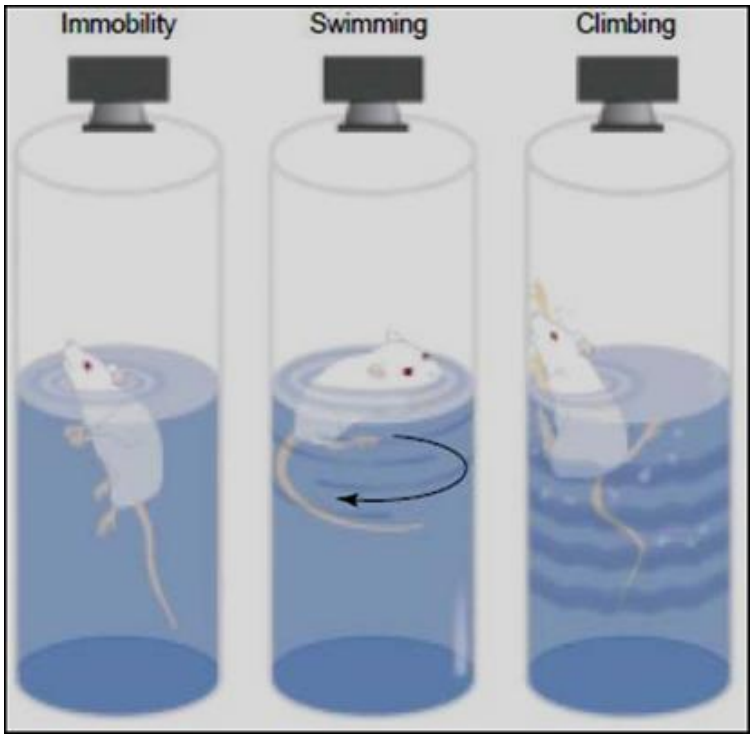

Figure A-3: The different behaviours observed in the FST (Cryan *et al.*, 2002).

The adapted FST is able to distinguish between serotonergic and noradrenergic mechanisms of antidepressants, as antidepressants that enhance serotonergic neurotransmission selectively increase the time spent swimming and antidepressants that enhance noradrenergic neurotransmission selectively increase the time spent struggling/climbing in the FST (Cryan *et al.*, 2002). Moreover, nearly all antidepressants decrease the time spent immobile in the FST (Cryan *et al.*, 2002). Diving behaviour (also classified as an escape-directed behaviour) and head shaking behaviour observed in the FST are generally not included in the behavioural analysis of the animal due to the episodic nature of these behaviours and these behaviours do not appear to correspond with specific treatment effects (Cryan *et al.*, 2005b). Therefore, diving behaviour was also excluded from the depressive-like behavioural analysis of animals in the FST during this study. A significant shortcoming of the acute FST is that short-term antidepressant treatment also effectively reduces the time animals spend immobile in the FST, whereas antidepressant treatment of several weeks is required to be effective in humans (Cryan *et al.*, 2002; Cryan *et al.*, 2005b; Overstreet & Wegener, 2013).

The FST apparatus used in this study consisted of four cylindrical tanks (40 cm (*h*) x 20 cm (*d*)) positioned next to each other and filled with water (30 cm deep and at a temperature of 25 ± 1°C). As previously mentioned, all behavioural tests were conducted during the dark cycle (i.e. between 18:00 and 06:00). Each rat was placed individually into a cylinder filled with water and allowed to swim for 5 min. The behaviour of the rats in the FST was recorded with a video

camera situated in front of the apparatus and was subsequently scored from the video recordings by investigators blind to the different treatment groups.

A.4 Methods for the BDNF analysis

On PnD 61, rats were euthanised by decapitation, brain samples were collected and the BDNF concentrations (i.e. a neuromarker of neuroplasticity and depression) in the hippocampi of the rats were measured. The BDNF analyses were conducted with rat BDNF enzyme-linked immunosorbent assay (ELISA) kits purchased from Elabscience Biotechnology Incorporated. The BDNF-analyses were conducted according to the instructions of the manufacturer. The complete method that was used for the BDNF analyses in this study can be viewed at https://www.elabscience.com/PDF/Cate61/E-EL-R1235-Elabscience.pdf.

Brain sample preparation:

- Firstly, the brain samples were weighed (i.e. each rat had one brain sample and all brain samples were analysed separately) and the volume of PBS (0.01M, pH=7.4) that needed to be added to each brain sample was calculated (tissue weight (g): PBS volume (ml) = 1:9).
- Then, the PBS was added to the brain samples and the suspensions were homogenised.
- Finally, the homogenates were centrifuged for 5 min at 5000×g to get the supernatant.

Reagent preparation

- Allow reagents to reach room temperature (i.e. 18-25°C) before use and preheat the Microplate reader for 15 min before measuring the optical density.
- Dilute 30 ml of the concentrated wash buffer with deionized water to produce 750 ml of the wash buffer.
- Centrifuge the standard at 10 000×g for 1 min and add 1 ml of the reference standard and sample diluent.
- Let it stand for 10 min and then turn it upside down for a few times.
- Mix thoroughly with a pipette (make sure it has fully dissolved). The stock solution produced from this reconstitution has a concentration of 2 000 pg/ml.
- Make serial dilutions as needed.
- Dilution method:
    - Take seven 1.5 ml eppendorf tubes and add 500 µL of reference standard, as well as 500 µL of sample diluent to each tube.

- Pipette 500 µL of the 2 000 pg/ml stock solution (from earlier) to the first tube and mix up to produce a stock solution with a concentration of 1 000 pg/ml.
- Pipette 500 µL of the solution from the former tube to the latter tube in order according to this step (see **Figure A-4**). Importantly, the last tube is considered a blank. Do not pipette solution into it from the former tube.

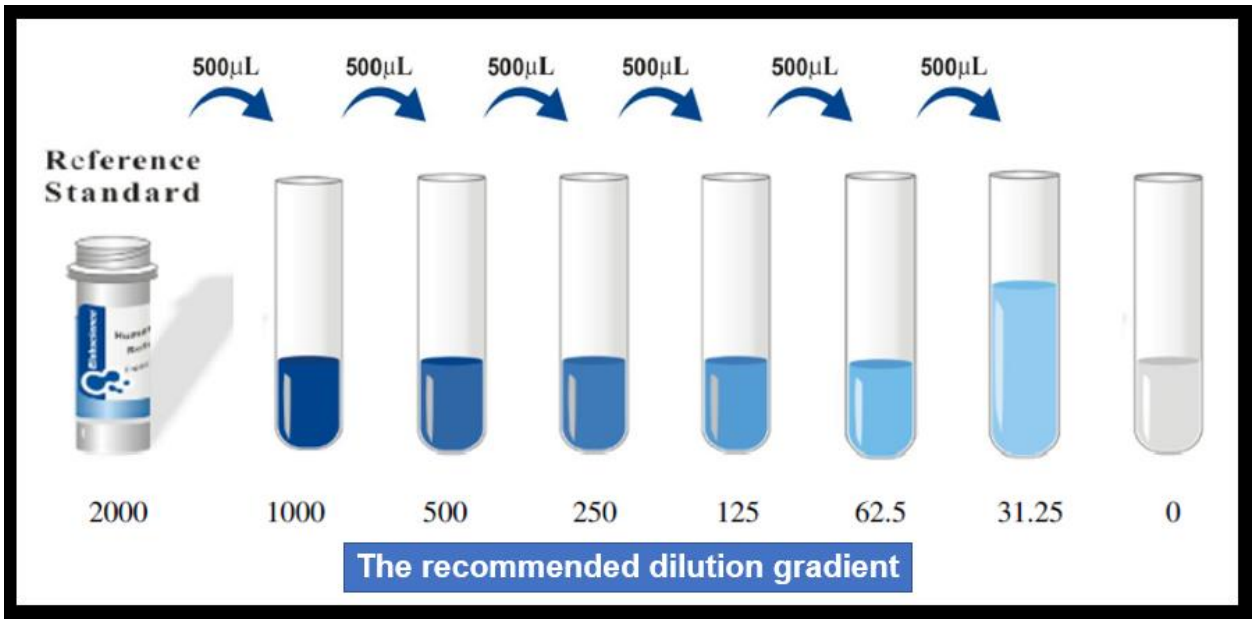

Figure A-4: An illustration of the dilution method. Adapted from Elabscience® Rat BDNF (Brain Derived Neurotrophic Factor) ELISA Kit (Catalog No: E-EL-R1235).

- Calculate the required amount of *Biotinylated Detection Ab working solution* before the experiment (100 µL/well).
- In the actual preparation, more account of 100-200 µL should be prepared.
- Centrifuge the stock tube before use.
- Dilute the 100X *Concentrated Biotinylated Detection Ab* to 1X working solution with *Biotinylated Detection Ab Diluent*.
- Calculate the required amount of *Concentrated HRP Conjugate working solution* before the experiment (100µL/well).
- In actual preparation, more account of 100-200 µL should be prepared.
- Dilute the 100X *Concentrated HRP Conjugate* to 1X working solution with *Concentrated HRP Conjugate Diluent*.

Assay procedure:

- Add 100 µL standard or sample to each well.
- Incubate for 90 min at 37°C.
- Remove the liquid.

- Add 100 µL *Biotinylated Detection Ab*.
- Incubate for 1 hour at 37°C.
- Aspirate and wash 3 times.
- Add 100 µL *HRP Conjugate*.
- Incubate for 30 min at 37°C.
- Aspirate and wash 5 times.
- Add 90 µL *Substrate Reagent*.
- Incubate for 15 min at 37°C.
- Add 50 µL *Stop Solution*.
- Read at 450 nm immediately.
- Calculation of results.

Addendum B: Additional results

This addendum contains additional data not presented in *Chapter 3*. The main aim of this study (see section 1.3) was to investigate the later-in-life bio-behavioural and cognitive effects of early-life (i.e. pre-pubertal and pubertal) exposure to a phosphodiesterase type 5 (PDE5) inhibitor (i.e. sildenafil) as observed in adulthood in stress sensitive Flinders Sensitive Line (FSL) rats. The FSL rat and a behavioural control, i.e. the Sprague-Dawley (SD) rat, were used in this study to investigate the later-in-life bio-behavioural and cognitive effects of sildenafil on rats with a genetic predisposition to develop major depressive disorder (MDD), i.e. the FSL rat, compared to a behavioural control, i.e. the SD rat, without a genetic predisposition to develop MDD.

B.1 Body weight

The rats were weighed daily from PnD 21 to 61. The results from the body weight analyses are discussed below. **Figure B-1** below depicts the body weight of the saline- and sildenafil-treated SD and FSL rats from PnD 21 to 61.

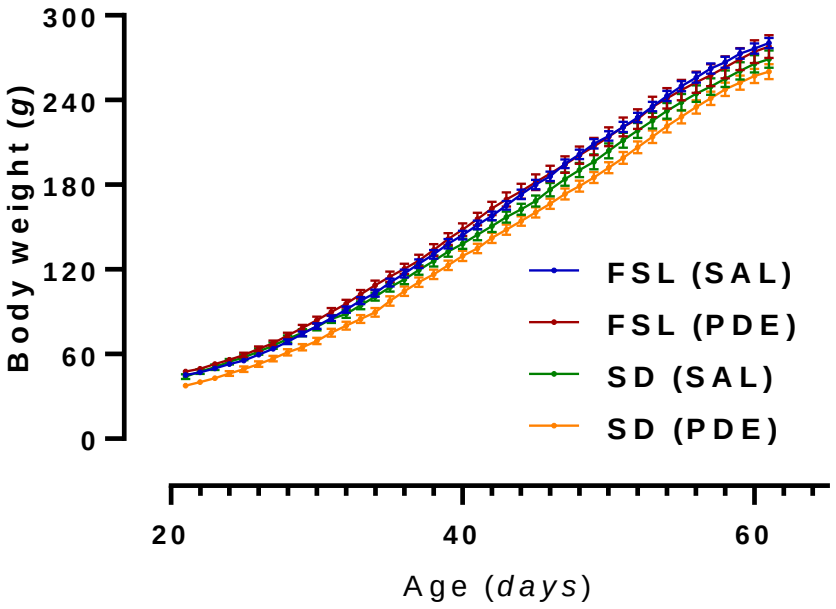

Figure B-1: Body weight of saline- and sildenafil-treated SD and FSL rats from PnD 21 to 61.

There were significant differences in initial body weight (i.e. PnD 21) between the different test groups ($F_{3.94} = 24.4$, $p < 0.0001$) (*data not shown*). Regardless, strong positive correlations between age and weight gain existed for all treatment groups ($r > 0.9$). Significant differences between the slopes of the regression lines for the different treatment groups was also identified ($F_{3.3928} = 14.0$, $p < 0.0001$) (FSL-SAL: $y = 6.40x – 107.0$; FSL-SIL: $y = 6.19x – 97.0$; SD-SAL: $y = 5.99x – 96.4$; SD-SIL: $y = 5.95x – 104.0$), suggesting overall differences in body weight gain rate. Of note, the slopes of the regression lines for the SD rats were comparable ($F_{1.1964} = 0.282$, $p = 0.595$), yet significantly differed for FSL rats ($F_{1,1964} = 6.640$, $p = 0.010$), suggesting that SIL-treatment significantly reduced body weight gain only in FSL rats, despite having similar PnD 21 body weight ($t_{39.7} = 1.60$, $p = 0.149$) (*data not shown*). Nevertheless, no significant body weight differences on PnD 61 were identified between any of the treatment groups, despite the Kruskal-Wallis test suggesting the medians to be significantly different ($F_{3.94} = 8.63$, $p = 0.035$) (*data not shown*).

B.2 BDNF concentrations in the hippocampi

Figure B-2 below depicts the later-in-life effects of early-life vehicle control and sildenafil treatment on hippocampal BDNF concentrations of adult SD and FSL rats.

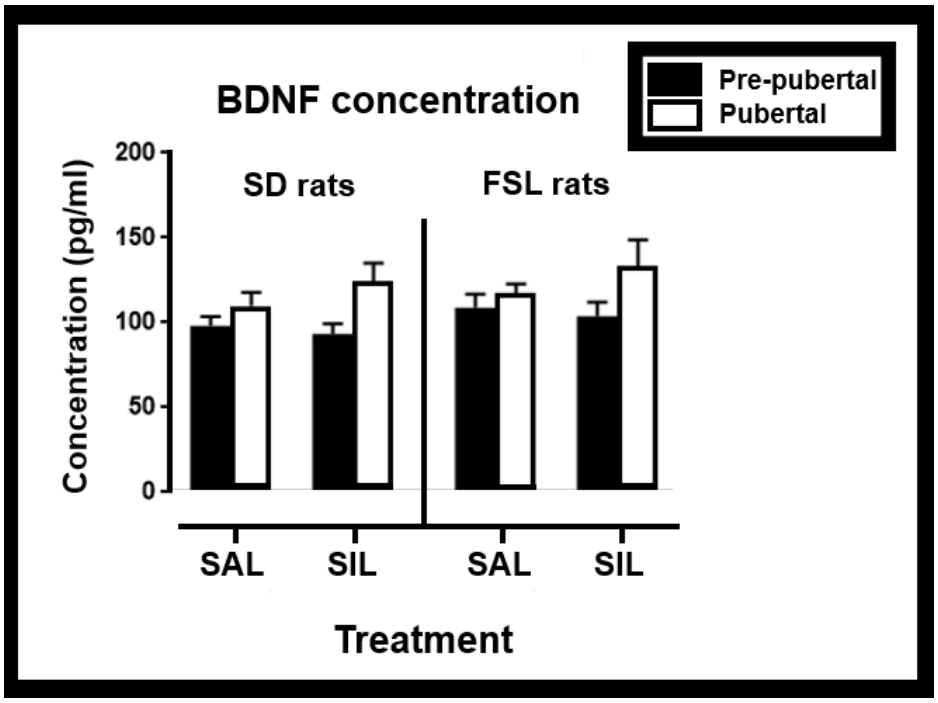

Figure B-2: Effects of sub-chronic pre-pubertal (PnD 21 – 34) and pubertal (PnD 35 – 48) vehicle control and sildenafil treatment on BDNF concentrations in the hippocampi of SD and FSL rats in early adulthood (PnD 60). BDNF concentrations measured in the hippocampi on PnD 61 following treatment of SAL+SD (n = 24), SAL+FSL (n = 24), SIL+SD (n = 24) or SIL+FSL (24). Data points represent the mean ± SEM. With abbreviations: SAL = saline, SIL = sildenafil, FSL = Flinders Sensitive Line rats, SD = Sprague-Dawley rats and BDNF = brain-derived neurotrophic factor.

In **Figure B-2**, there was no statistically significant three-way interaction between treatment, age and strain ($F_{1.88}$ = 0.006170, p = 0.9376, η^2 = 0.007) for brain-derived neurotrophic factor (BDNF) concentrations in the hippocampi on PnD 60, nor any statistically significant two-way interactions.

BDNF is one of several neurotrophic factors found in the human brain and plays a pivotal part in MDD and cognitive function (Pittenger & Duman, 2008; Hasselbalch *et al.*, 2012; Serafini, 2012). MDD is known to present with reduced levels of BDNF (Fakhoury, 2016) that is reversible with antidepressant treatment (Hasselbalch *et al.*, 2012). In this regard, extensive reviews previously conducted substantiate the antidepressant-related, e.g. SSRIs (selective serotonin reuptake inhibitors), MOAIs (monoamine oxidase inhibitors), TCAs (tricyclic antidepressants), electroconvulsive therapy (ECT) and ketamine, reversal of cognitive (i.e. learning and memory) and neuroplasticity deficits in both preclinical and clinical studies (Pittenger & Duman, 2008; Serafini, 2012). Section 2.5.1.4 elaborates more on the implication of BDNF in both MDD and cognition.

Unfortunately, there was no statistically significant effect on hippocampal BDNF concentrations of rats on postnatal day (PnD) 60 following pre-pubertal and pubertal sildenafil treatment in the

current study. This may be due to the transient enhancement of neuroplasticity observed following early-life antidepressant treatment. In this regard, a recent study in our laboratories showed that pre-pubertal escitalopram (i.e. an SSRI) treatment significantly increases hippocampal BDNF concentrations in FSL rats when measured on PnD 35 (i.e. immediately following escitalopram treatment), however when measured on PnD 60 (i.e. after a sub-chronic "wash-out" period), the increased hippocampal BDNF concentrations observed in FSL rats returned to baseline levels, as no statistically significant difference in hippocampal BDNF concentrations is observed between escitalopram-treated and vehicle-treated FSL rats on PnD 60 (Steyn *et al.*, 2018). Furthermore, BDNF is strongly associated with cognition (Griesbach *et al.*, 2009) and this could explain the lack of improvement in memory observed in FSL rats on PnD 60 in the nORT.

Addendum C: Congress proceedings

This addendum contains an abstract of data that were presented at a national congress in 2018 as well as proof of attendance.

The results of this study were presented as a podium presentation for the Young Scientist competition of the South African Society for Basic and Clinical Pharmacology 2018, held in Stellenbosch, South Africa.

C.1 Abstract

Juandré L.B. Saayman, Stephanus F. Steyn, Francois P. Viljoen, Christiaan B. Brink. Long-lasting behavioural effects of early-life sildenafil treatment in stress-sensitive versus healthy control rats. The first annual Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST), Stellenbosch (07 – 10 October 2018).

Background:

Juvenile depression is a tremendous concern globally, with only fluoxetine and escitalopram approved for treatment. A rise in the prevalence of depression and in the number of prescriptions for antidepressants have been observed in juveniles. A delayed onset of action, high rates of relapse and low remission rates are frequently seen with juvenile antidepressant treatment and potential long-lasting effects of psychotropic drugs on neurodevelopment are unclear. Therefore, novel antidepressants are needed, along with research on their potential long-lasting effects. This study investigated the long-lasting effects of early-life chronic sildenafil treatment on measures of depressive-like behaviour and cognition in adulthood and the age at which sildenafil treatment has the most notable effects, viz. pre-pubertal, pubertal or both.

Materials and methods:

Male Flinders sensitive line and Sprague-Dawley rats received either sildenafil (3 mg/kg/day) or vehicle-control subcutaneously from postnatal day 21 to 34 (pre-puberty) or from postnatal day 35 to 48 (puberty). On postnatal day 60 (adulthood), the rats were subjected to the novel object

recognition test, the open field test and the forced swim test to assess cognition, locomotor activity and anxiety-like behaviour, as well as antidepressive-like behaviour, respectively.

Results:

Juvenile sildenafil treatment had no effect on cognition, locomotor activity or anxiety-like behaviour in both strains and regardless of the age of treatment. In the forced swim test, the Flinders sensitive line rats displayed greater immobility than the Sprague-Dawley rats and following sildenafil treatment the immobility of these rats was reduced, regardless of the age of treatment. In addition, sildenafil treatment increased struggling behaviour in the Flinders sensitive line rats, regardless of the age of treatment and increased swimming behaviour in the pre-pubertal treated groups, regardless of the strain.

Conclusions:

Early-life sub-chronic sildenafil treatment has long-lasting antidepressive-like effects into adulthood, with no effect on cognition and anxiety-like behaviour.

C.2 Proof of attendance

This serves to confirm that

Juandré Saayman

attended the

First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) 2018

at

Spier Conference Centre, Stellenbosch, and Cape Town, South Africa

Prof Helmuth Reuter
Chair: Organising committee

Addendum D: Ethics approval letter

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Email : Ethics@nwu.ac.za

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **AnimCare Animal Research Ethics Committee (AREC-130913-015)** on **01/09/2017** after being reviewed at the meeting held on **22/06/2017**, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Long-lasting bio-behavioural effects of early-life sildenafil administration in a genetic rat model of depression

Study Leader/Supervisor: **Prof Christiaan B Brink**
Student: Mr Juandré Saayman

Ethics number:

N	W	U	-	0	0	2	7	7	-	1	7	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission, P = Provisional Authorisation, A = Authorisation

Application Type: New Application – Standard Project

Commencement date: 2017-09-01 **Category:** 4

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.

Special conditions of the approval (if applicable):

- Any permits/ permission must still be obtained from relevant authorities and provided to the AnimCare, Faculty of Health Sciences. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via AnimCare:*
  - *annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the AnimCare, prior to implementation. Would there be deviated from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-IRERC and AnimCare retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the AnimCare or that information has been false or misrepresented,*
    - *the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *AnimCare can be contacted for further information or any report templates via Ethics-AnimCare@nwu.ac.za or 018 299 2197.*

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the IRERC or AnimCare for any further enquiries or requests for assistance.

Yours sincerely

Prof LA Du Plessis
Digitally signed by Prof LA Du Plessis
Date: 2017.09.07 07:57:59 +02'00'

Prof Linda du Plessis
Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)