

# The effect of early-life exposure of rats to venlafaxine on behaviour and neurological markers of antidepressant action in adulthood

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# Abstract

Major depression is a serious mood disorder affecting more than 120 million people worldwide, irrespective of their race or socio-economic status. This psychiatric disorder is predicted to become the second leading cause of disability by the year 2020, second only to heart diseases in the global population, without distinguishing differences in the incidence within defined age groups. Depression is known to affect people across all age groups, including children, adolescents, adults and geriatrics, although older age is associated with an increased susceptibility to major depression and other psychiatric conditions. Until the 1970's depression during childhood and adolescence was thought to be uncommon or non-existent. Recent epidemiological studies have demonstrated that there is a persistent escalation in the prevalence of depression in children and adolescents. Accordingly, the number of prescriptions for drugs to treat this disorder in juveniles has escalated significantly. With our current limited understanding of the safety and long-term effects of treatment with antidepressants, the clinician is left making decisions without sound evidence of safety. In addition, psychotropic drugs may affect neurodevelopment during childhood and adolescence and may consequently modulate susceptibility to psychiatric disorders later in life.

The objective of the current study was to investigate the effects of early-life (pre-natal and postnatal) chronic treatment with venlafaxine, a dual action serotonin-noradrenalin reuptake inhibitor, during the developmental phase of the serotonin and norepinephrine pathways in stress-sensitive rats on measures of cognition, anxiety-like and depressive-like behaviour later in life. The study also investigated which age shows optimal behavioural changes later in life, following the above mentioned administration of venlafaxine. In addition we also determined the effects that the administration of venlafaxine has on the levels of monoamines *l*-norepinephrine (*l*-NE) and serotonin (5-HT) in the prefrontal cortex and the hippocampus.

A number of translational animal models of psychiatric disorders have been described and validated, and is suitable for such investigations. For the current study we used stress-sensitive Flinders Sensitive Line (FSL) rats and their controls, Flinders Resistant Line (FRL) rats. Pregnant dams were injected subcutaneously for 14 days with 10 mg/kg venlafaxine or saline from pre-natal day 15 (ND-15) to ND-01. New-born pups were then injected subcutaneously with 3 mg/kg venlafaxine or saline for 14 days from postnatal day 3 (ND+03) to ND+17. These doses were determined from previous studies reported in literature. Four rat treatment groups of both FSL and FRL rats received injections during pre-natal + postnatal ages as follows: saline + saline, venlafaxine + saline, saline + venlafaxine and venlafaxine + venlafaxine. Following the drug treatments, all rat groups were housed under normal conditions until the indicated time to be subjected to a battery of behavioural tests, including the novel object recognition test (nORT), locomotor activity test (Digiscan®), elevated plus maze (EPM) and forced-swim test (FST), scheduled on either ND+35, ND+60 or ND+90. Separate treatment groups were used for each age group. After the behavioural tests animals were decapitated, the brains removed and the prefrontal cortex and hippocampus dissected out. These were analysed at a later stage using an HPLC with electrochemical detection to determine the levels of the monoamines *I*-NE and 5-HT. All animal procedures were approved by the Ethics Committee of the North-West University (approval number: NWU-00045-10-S5), and are in accordance with the recommendations of the National Institutes of Health guide for the care and use of laboratory animals.

The data from the current study suggest that in general FRL rats were not influenced by the early-life treatment with venlafaxine, as observed in the nORT, EPM or FST on ND+35, ND+60 or ND+90. There was minimal changes seen in the immobile behaviour in the FST of FRL rats that received prenatal venlafaxine. As expected, depressive-like behaviour in the FST was significantly enhanced in FSL rats relative to corresponding FRL rat groups as observed at ND+35 and ND+60, but not ND+90. Importantly, depressive-like behaviour was reversed following pre- and postnatal treatment with venlafaxine in FSL rats at ND+60, relative to the corresponding FRL rat

groups. Reversal of depressive-like behaviour in FSL rats were not observed at ND+35 or ND+90, suggesting a delayed response that is reversed later in adulthood. The data from the nORT, Digiscan® or EPM did not reveal any significant differences between the various FSL treatment groups, including at ND+60.

The current study therefore demonstrated that the treatment regimen employed had a transient effect on depressive-like behaviour later in life and suggested that genetic susceptibility plays an important role in the treatment of depression. This was suggested by the venlafaxine-induced decrease in immobile behaviour exhibited by FSL rats at ND+60 in the FST, and the subsequent increase in immobile behaviour at ND+90. In general, the most significant venlafaxine-induced effects were seen in FSL rats, suggesting genetic susceptibility plays an important role.

**Keywords:** Depression, Children and Adolescents, 5-HT, Serotonin, I-NE, Norepinephrine, Venlafaxine, Behaviour, Forced Swim Test.

# Uittreksel

Major depressie is 'n ernstige gedragsafwyking wat meer as 120-miljoen mense wêreldwyd affekteer, ongeag van ras of sosio-ekonomiese status. Dit is voorspel dat hierdie psigiatriese afwyking die tweede grootste oorsaak van gestremdheid teen die jaar 2020 sal wees, en dan tweede slegs na hartsiektes in die globale populasie, sonder om verskille in insidensie te onderskei binne gedefinieerde ouderdomsgroepe. Dit is alom bekend dat depressie mense van alle ouderdomsgroepe affekteer, insluitend kinders, adolessente, volwassenes en bejaardes, alhoewel gevorderde ouderdom geassosieer word met 'n verhoogde vatbaarheid tot major depressie en ander psigiatriese toestande. Tot die 1970's is geglo dat depressie onder kinders en adolessente seldsaam of selfs afwesig is. Onlangse epidemiologiese studies het aangetoon dat daar 'n volgehoue verhoging in die voorkoms van depressie in kinders en adolessente is. Gevolglik het die aantal voorskrifte vir geneesmiddels vir die behandeling van van hierdie afwyking onder die jeug beduidend toegeneem. Met ons huidige beperkte begrip van die veiligheid en die langtermyn effekte van die behandeling met antidepressante, word die klinikus gelaat om besluite sonder behoorlike bewyse van veiligheid te neem. Verder kan psigotropiese middels neurologiese ontwikkeling gedurende die kinderjare en adolessensie beïnvloed en mag dit gevolglik vatbaarheid vir psigiatriese versteurings later in die lewe moduleer.

Die doel van die huidige studie was om die effek van chroniese behandeling vroeg in die lewe (pre- en postnataal) met venlafaksien, 'n middel met tweeledige werking om serotonien en noradrenaliniese heropname in te hê, te ondersoek. Venlafaksien is sodoende toegedien gedurende die ontwikkelingsfase van die serotonien- en norepinefrienbane in stressensitiewe rotte, en geëvalueer vir gevolglike effekte op kognisie, angs-agtige en depressief-agtige gedrag later in die lewe. Die studie het ondersoek ingestel na die ouderdom waartydens die mees prominente gedragsveranderinge gesien word later in die lewe, soos dit voorkom na die voorafvermelde toediening van venlafaksien. Daar benewens het ons ook die effekte van die toediening van venlafaksien op die vlakke van die

monoamiene *l*-norepinefrien (*l*-NE) en serotonien (5-HT) in die prefrontale korteks en die hippokampus bepaal.

'n Aantal translasie-dieremodelle van psigiatriese afwykings is al beskryf en gevalideer, en is bruikbaar vir sodanige ondersoeke. In hierdie studie het ons Flinders se Sensitiewe Lyn- (FSL-) rotte en hulle kontrole lyn die Flinders se Weerstandige Lyn- (FWL-) rotte gebruik. Die dragtige rotte is vir 14 dae met 10mg/kg venlafaxine of met soutoplossing vanaf prenatale dag 15 (ND-15) tot ND-01 subkutaan ingespuut. Die nuutgebore rot-kleintjies is met 3 mg/kg venlafaxine of met soutoplossing vir 14 dae vanaf ND+03 tot ND+17 subkutaan ingespuut. Hierdie dosisse is vasgestel vanuit vorige studies wat in die literatuur gerapporteer is. Vier rot behandelingsgroepe van beide die FSL- en FWL-rotte het inspuutings gedurende pre-natale en postnatale ouderdomme ontvang: soutoplossing + soutoplossing, venlafaksien + soutoplossing, soutoplossing + venlafaksien en venlafaksien + venlafaksien. Na die geneesmiddelbehandelings is al die rotgroepe onder normale omstandighede aangehou tot die aangeduide tyd vir onderwerping aan 'n battery van gedragstoetse, insluitend die nuwe voorwerpherkenningstoets (nVHT), die lokomotor-aktiwiteitstoets (Digiscan®), die verhewe plus-doolhof (VPD) en die geforseerde swemtoets (GST), geskeduleer op ND+35, 60 of 90. Verskillende behandelingsgroepe is vir elke ouderdomsgroep gebruik. Na afloop van die gedragstoetse is die diere gedekapiteer, die breine is verwyder en die prefrontale korteks en hippokampus uitgedissekteer. Hierdie breindele was later geanaliseer deur gebruikmaking van 'n hoëdruk vloeistof kromatograaf (HDVK) met elektrochemiese deteksie om die vlakke van die monoamiene *l*-NE en 5-HT te bepaal. Alle prosedures wat op die diere uitgevoer is, was deur die Etiese Komitee van die Noordwes-Universiteit goedgekeur (goedkeuringsnommer: NWU-00045-10-S5), en volg die riglyne wat deur die Nasionale Instituut van Gesondheid vir die sorg en gebruik van laboratoriumdiere daargestel is.

Die data wat tydens die studie versamel is, het in breë trekke aangedui dat FWL-rotte nie deur jeugdige behandeling met venlafaksien beïnvloed is nie, soos waargeneem in die nVHT, VPD of die GST teen ND+35, ND+60 of

ND+90. Daar was egter minimale veranderinge in immobiele gedrag waarneembaar in die GST van van FWL-rotte wat prenataal venlafaksien ontvang het. Soos verwag is die depressie-agtige gedrag in die FSL-rotte beduidend relatief to die ooreenstemmende FWL-rotte soos waargeneem teen ND+35 en 60, maar nie ND+90 nie. Dit is belangrik om op te merk dat depressie-agtige gedrag omgekeer is na pre- en postnatale toediening van venlafaksien in FSL-rotte teen ND+90. Omkering van depressie-agtige gedrag in FSL-rotte omkering is nie waargeneem teen ND+35 of ND+90 nie en dit veronderstel dat hierdie 'n vertraagde respons wat later in die volwasse lewe omgekeer word. Die data van die nVHT, Digiscan® en VPD het geen betekenisvolle veranderinge tussen die onderskeie FSL behandelingsgroepe aangetoon nie, insluitend teen ND+60.

Die huidige studie het dus getoon dat die behandelingsregime wat gebruik is 'n verbygaande effek gehad het op depressie-agtige gedrag en stel ook voor dat genetiese vatbaarheid 'n groot rol speel in die behandeling van depressie. Dit is aangedui deur die venlafaksien-geïnduseerde verlaging in immobiele gedrag wat in FSL-rotte teen ND+60 waargeneem is, en die daaropvolgende verhoging in immobiele gedrag teen ND+90. Oor die algemeen was die mees prominente effekte in FSL rotte waargeneem, wat aandui dat genetiese vatbaarheid 'n belangrike rol speel.

**Sleutelwoorde:** Depressie, Kinders en Adollesente, 5-HT, Serotonien. I-NE, Norepinifrien, Venlafaksien, Gedrag, Geforseerde Swem Toets.

“Work hard in silence,  
let success be your noise”  
-Frank Ocean

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“Arise and be all that you dreamed” – Flyleaf

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# List of Abbreviations

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**Numerals** 5-HT 5-Hydroxytryptophan (Serotonin)

---

5-HT<sub>x</sub> 5-Hydroxytryptophan (Serotonin) -  
receptor subtype

---

α<sub>x</sub> Alpha -receptor subtype

---

**A**

AAFP American Academy of Family  
Physicians

---

ACh Acetylcholine

---

AChE Acetylcholinesterase

---

ACTH Adrenocorticotropin

---

**B**

BDNF Brain derived neurotrophic factor

---

**C**

CNS Central nervous system

---

CREB Cyclic adenosine monophosphate  
response element binding protein

---

CRH Corticotropin-releasing hormone

---

---

## D

D Dopamine -receptor subtype

---

DA Dopamine

---

DAAM Digiscan® animal activity monitor

---

DFP Diisopropyl fluorophosphates

---

DI Discrimination index

---

DNA Deoxyribonucleic acid

---

DSM-IV Diagnostic and Statistical Manual of Mental Disorders, 4th edition

---

## E

ECT Electroconvulsive therapy

---

EPM Elevated Plus-maze

---

ER Extended release

---

---

## F

FDA Food and Drug Administration

---

FRL Flinder's Resistant Line

---

FSL Flinder's Sensitive Line

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FST Forced Swim Test

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## H

H<sub>x</sub> Histamine -receptor subtype

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HIV Human Immunodeficiency Virus

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HPA-axis Hypothalamic-pituitary-adrenal-axis

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## L

/-NE Norepinephrine

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## M

MAO Monoamine oxidase

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MAOI Monoamine oxidase inhibitor

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MD Major depression

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MDD Major depressive disorder

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MDE Major depressive episode

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MHRA Britain's Medicines and Healthcare  
products Regulatory Authority

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MPFC Medial prefrontal cortex

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## N

nAChRs Nicotinic acetylcholine receptors

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NET Norepinephrine transporter

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nORT Novel object recognition test

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NE Norepinephrine

---

ND+ Postnatal day

---

ND- Prenatal day

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## P

PDE 5 Phosphodiesterase 5

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PFC Prefrontal cortex

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PVN Paraventricular nucleus

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## R

rCBF Regional cerebral blood flow

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REM Rapid eye movement

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RSA Republic of South Africa

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# S

Sal Saline

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s.c. Subcutaneously

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SERT Serotonin transporter

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SNRI Serotonin-norepinephrine reuptake inhibitor

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SSRI Selective serotonin reuptake inhibitor

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# T

TCA Tricyclic antidepressant

---

# U

USA United States of America

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# V

Ven Venlafaxine

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# W

WHO World Health Organization

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# Chapter 1 Introduction

## 1 Introduction

This chapter is included as an orientation to the current study and dissertation, containing a condensed summary of the study. A more complete literature review follows in Chapter 2. Chapter 3 contains a more in-depth look at the materials and methods, Chapter 4 presents the results and discussion, and Chapter 5 summarises, concludes and makes recommendations. Appendix A is added to the dissertation with regard to the contributions made at the 3's Company Congress in Cape Town during the study.

### 1.1 Problem statement

Major depression is a serious mood disorder affecting more than 120 million people worldwide, irrespective of their race or socio-economic status. This psychiatric disorder is predicted to become the second leading cause of disability by the year 2020, second only to heart diseases (World Health Organisation, 2011a) in the global population, without distinguishing differences in the incidence within defined age groups. Depression is known to affect people across all age groups, including children, adolescents, adults and geriatrics, although older age is associated with an increased susceptibility to major depression and other psychiatric conditions (Harrington et al., 1990; Lewinsohn et al., 2000).

In the 1970's the debate was still raging on whether depression could affect children (Malkesman & Weller, 2009) and epidemiological studies conducted since confirmed that they are indeed affected (Birmaher et al., 1996; Jane Costello et al., 2006; Keenan et al., 2004; Kessler et al., 2001). The number of children that are being diagnosed and treated for major depression has increased considerably during the last two decades. The cause for this marked increase has been ascribed not only due to better diagnosis, but to an increase in the incidence of anxiety-related disorders in children and adolescents in developing countries as well (Zito et al., 2002; Zito & Safer, 2001).

Data on the use of antidepressants in the treatment of depression in children and adolescents are limited, and the potential long term neuropsychiatric effects are mostly unknown. In the United States of America (USA) the Food and Drug Administration (FDA) has approved fluoxetine (Prozac®) and escitalopram (Lexapro®), both selective serotonin reuptake inhibitors (SSRI), as the drugs of choice for treatment of depression in children and adolescents (Bylund & Reed, 2007; Soutullo & Figueroa-Quintana, 2013; Wagner, 2005). Fluoxetine is approved for use in children and adolescents from 7-17 years of age and escitalopram for adolescents aged 12-17 years (Soutullo & Figueroa-Quintana, 2013). It is also required by legislation to carry the warning that it may cause an initial increase in suicidal thoughts and ideation (Wagner, 2005), which is considered potentially life-threatening.

It has been suggested in numerous studies that SSRIs are clinically more effective in the treatment of major depression in children and adolescents than drugs modulating the noradrenergic neurotransmission (Bridge et al., 2007; Hazell et al., 1995; Kratochvil et al., 2006; Mason et al., 2009; Whittington et al., 2004). This is different from the treatment of adults and is believed to relate to the fact that serotonergic neurodevelopment is mature before the onset of adolescence, whereas noradrenergic neurodevelopment matures only in early adulthood. In this regard, the tricyclic antidepressants (TCAs) class of antidepressants preferentially target the noradrenergic pathway, or in some cases both the serotonergic and noradrenergic pathways, whereas the SSRIs selectively target the serotonergic pathway (Choi et al., 2009; Findling & McNamara, 2004; Lewis, 1998; Murrin et al., 2007).

It is important to understand the long-term effects that the antidepressants may have on children and adolescents. The increase in the number of SSRI prescriptions for these age groups (Zito et al., 2002; Zito & Safer, 2001) necessitates a better understanding of the long-term effects of the drugs on neurodevelopment and the chances of relapse or the development of other psychiatric disorders later in life. The risk of acute side effects is currently outweighed by the benefit of using antidepressants to treat severe MDD (to counteract the serious symptomatology and risk of suicide). Preclinical

studies in rats suggest that exposure to psychotropic drugs early in life will induce neurochemical changes in the developing brain and that these changes will manifest only later in life (Choi et al., 2009; Noorlander et al., 2008). These outcomes are not well defined, but may be relevant also to humans, suggesting that early-life antidepressant treatment may potentially affect neurodevelopment and hence the psychiatric outcome later in life.

Furthermore, major depression is associated with genetic susceptibility, so that susceptible individuals are more likely to develop major depression than normal individuals. Individuals developing major depression early in life and hence receiving antidepressant treatment are more likely than those with genetic susceptibility to be affected by the treatment. This necessitates that studies investigating the effect of early-life exposure to antidepressants on outcome later in life, should factor this variable in.

Our laboratory therefore aims to determine the long-term neurobiological, neurobehavioural and cognitive effects of early-life exposure of stress sensitive rats to an antidepressant drug (venlafaxine, a serotonin-norepinephrine reuptake inhibitor), compared to a stress-resilient control line. In a previous study in our laboratory (Steyn, 2011) pregnant dams of stress-sensitive Flinders sensitive line (FSL) rats and their control line Flinders resistant line (FRL) rats were exposed to venlafaxine treatment or vehicle control at prenatal days 15 (or natal day -15; ND-15) to ND-01 (pregnant dams of venlafaxine groups receiving 10 mg/kg/day), and again on postnatal day 3 (ND+03) to ND+17 (pups of venlafaxine groups receiving 3 mg/kg/day). Thereafter rats were housed normally until ND+35, ND+60 and ND+90 when behavioural analyses were performed. From the results found in our laboratory by Stephan Steyn (Steyn, 2011) it was evident that ND+21 may be too early to detect significant changes in behaviour and that this age group may be excluded from future studies. In addition, it was recommended that ND+90 be investigated as a later point in life to investigate the long-term effects of early-life exposure. The current study, as described below, followed these guidelines. However, the previous study also lacked statistical power, so that additional animals were to be added to the treatment groups.

## 1.2 Study objectives

The objectives of this study were to:

- investigate whether early-life chronic administration of the SNRI venlafaxine to rats induces long-term effects manifesting as bio behavioural changes later in life, and if so to
- determine the optimal age for the detection of late-life bio behavioural changes, and to
- investigate the role of genetic susceptibility in stress-sensitive FSL versus control FRL rats in the development of late-life bio behavioural changes.

The study layout to achieve these objectives is described under Study Layout below. In particular, this study will measure the effects of early-life intervention on locomotor activity in the Digiscan® apparatus, anxiety-like behaviour in the elevated plus maze, memory consolidation in the novel object recognition test and depressive-like behaviour in the Forced swim test, as well as serotonin and norepinephrine levels in the frontal cortex and hippocampus.

## 1.3 Hypothesis and expected outcomes

It is postulated that early-life administration of venlafaxine will induce long-lasting effects, presenting as altered behaviour and changes in neurobiological markers later in life. Both the serotonergic and noradrenergic pathways undergo development during the early-life treatment period and affect neurodevelopment differentially differently (Bylund & Reed, 2007). Venlafaxine inhibits the reuptake of both serotonin and norepinephrine, and thus will target both these signalling pathways simultaneously. The study is therefore postulated to reveal any behavioural, cognitive and neurobiological changes via either of these two monoaminergic mechanisms. We postulate that early-life venlafaxine will positively affect neurodevelopment resulting in unchanged locomotor activity, reduced anxiety-like behaviour, enhanced cognition and reduced depressive-like behaviour later in life.

Furthermore, by employing the stress-sensitive FSL rat and its control line, the FRL rat, this study will reveal any role of genetic susceptibility to neurodevelopmental effects of early-life venlafaxine. We postulate that early-life administration of venlafaxine will yield prominent late-life effects (explained above) in FSL rats, but not in FRL rats.

In addition, the behavioural data from the forced swim test and the measurement of monoamine concentrations will hint towards neurological mechanisms that may underlie any neurodevelopmental effects of venlafaxine, in particular whether serotonergic and adrenergic mechanisms are involved. We postulate that early-life venlafaxine will enhance both serotonergic and adrenergic neurotransmission later in life. We believe that the results of the study will guide future studies evaluating long-lasting effects of the early-life administration of various psychotropic drugs, be it drugs selectively affecting noradrenergic or serotonergic neurotransmission or diverse mechanisms.

#### **1.4 Study layout**

To achieve these study objectives, we used stress sensitive Flinders sensitive line (FSL) and their controls, Flinders resistant line (FRL) rats. All rats received venlafaxine or vehicle control prenatally on ND-15 to ND-1 (pregnant dams administered 10 mg/kg/day subcutaneously; s.c.) and again postnatal as pups on ND+3 to ND+17 (3 mg/kg/day). Thereafter rats were housed normally until ND+35, ND+60 or ND+90, when behavioural analyses were performed and brain tissue were dissected for biochemical analyses. Here ND+35 represents the onset of sexual maturity (adolescence) (Murrin et al., 2007; Zeinoaldini, 2005), whereas ND+60 represents early adulthood and ND+90 later stage adulthood. The dosing regimen of venlafaxine for the treatment of pregnant dams and pups were selected based on previous studies. Pregnant dams received venlafaxine at a dose of 10 mg/kg s.c. (Folkesson et al., 2010; Larsen et al., 2010; Scaini et al., 2010), whereas the pups received a dose of 3 mg/kg (s.c.) (Dawson et al., 1999).

The basic study layout is presented in Table 1-1 below:

**Table 1-1:** Basic layout of the study, indicating the various treatment groups

<b>Rat line</b>	<b>Pre-natal administration</b>	<b>Postnatal administration</b>	<b>Late-life testing</b>
<b>FSL</b>	Vehicle control	Vehicle control	ND+35
	Venlafaxine	Vehicle control	
	Vehicle control	Venlafaxine	
	Venlafaxine	Venlafaxine	
<b>FRL</b>	Vehicle control	Vehicle control	ND+35
	Venlafaxine	Vehicle control	
	Vehicle control	Venlafaxine	
	Venlafaxine	Venlafaxine	
<b>FSL</b>	Vehicle control	Vehicle control	ND+60
	Venlafaxine	Vehicle control	
	Vehicle control	Venlafaxine	
	Venlafaxine	Venlafaxine	
<b>FRL</b>	Vehicle control	Vehicle control	ND+60
	Venlafaxine	Vehicle control	
	Vehicle control	Venlafaxine	
	Venlafaxine	Venlafaxine	
<b>FSL</b>	Vehicle control	Vehicle control	ND+90
	Venlafaxine	Vehicle control	
	Vehicle control	Venlafaxine	
	Venlafaxine	Venlafaxine	
<b>FRL</b>	Vehicle control	Vehicle control	ND+90
	Venlafaxine	Vehicle control	
	Vehicle control	Venlafaxine	
	Venlafaxine	Venlafaxine	

Since the current study followed up on a previous study lacking statistical power, data from this previous study was combined with the current study, aiming to have a total of 12 rats per treatment group.

Late-life evaluations included behavioural and cognitive testing, and biochemical testing of brain tissue. The behavioural and cognitive testing was implemented as a battery of tests in the following order:

- Digiscan® animal activity monitor (locomotor activity)
- novel object recognition test (cognition)
- elevated plus maze (anxiety-like behaviour)
- forced swim test (depressive-like behaviour)

- However, the previous study also lacked statistical power, so that additional animals were to be added to the treatment groups where optimal changes in anxiety-like and depressive-like behaviour and cognition is observed after pre- and/or postnatal exposure to venlafaxine (an SNRI antidepressant drug that inhibits the reuptake of serotonin and noradrenaline). These changes in behaviour will be determined by performing a battery of behavioural tests. The tests will measure different parameters of behaviour as well as brain function on the rats at different stages later in their life. Parameters that will be measured include cognitive activity, locomotor activity and also depressive-like and anxiety-like behaviour.
- Determining the effect of venlafaxine on the neurobiological markers of antidepressant action, serotonin and *l*-norepinephrine, in the prefrontal cortex and hippocampus of the rat brain.
- Determining whether stress sensitive (FSL) rats respond differently to treatment than their control line, the Flinders resistant line (FRL) rat.

The specific ages of ND+35, 60 and 90 were chosen to replicate specific stages in the developmental process of the animal. ND+35 represents the adolescent stage, as sexual maturity occurs during the fifth week after birth (Murrin et al., 2007; Zeinoaldini, 2005), whereas ND+60 represents an early stage in adulthood and ND+90 a later stage in adulthood.

## **1.5 Ethical approval**

All animal procedures are in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals and was approved by the Ethics Committee of the North-West University (approval number: NWU-00045-10-S5).

## **1.6 Dissertation approach and layout**

This dissertation will be written and submitted in the standard format for thesis/dissertation submission at the North-West University. The dissertation will consist of five chapters and appendixes as explained in the chapter.

# Chapter 2 Literature Background

## **2 Literature background**

In light of the study objectives, the current chapter will set out to give a literature review discussing general characteristics of major depression (epidemiology, neurobiology, neurodevelopment, hypotheses, diagnosis, signs and symptoms and treatment options for major depression). The review will also focus on the treatment of children and adolescents with major depression, reflect on previous clinical and pre-clinical studies on the topic, provide a theoretical framework of long-lasting drug effects, and finally summarise with a synopsis.

### **2.1 Major depressive disorder**

Depression is best described as a transient state of mood that is experienced by virtually all people at some time in their lives. This may be a normal response to stressful events that happen in their lives, but may also become a serious clinical disorder (Bylund & Reed, 2007). When these symptoms present as a persistent and debilitating clinical disorder, even in the absence of direct causal circumstances or events, the condition is referred to as Major Depressive Disorder (MDD). The ability of the affected person to function in normal daily life is adversely affected and symptoms include feelings of hopelessness and worthlessness with a persistent sad or “empty” mood. Changes in sleep and appetite, loss of interest in pleasurable activities, concentration difficulties, deficits in decision making and memory, and thoughts of suicide or death are also prevalent (Fava & Kendler, 2000).

MDD is one of the oldest and best described medical disorders, first described in ancient Greek medical texts (Fava & Kendler, 2000). It was only as late as the mid-1960s that MDD was acknowledged as a biochemical phenomenon. In recent times it has been estimated that MDD is the most costly brain disorder in Europe. The total cost of the disorder corresponds to 1% of the total annual European economy (Sobocki et al., 2006). It was estimated in a

European study in 2004 that the total cost of depression is around 118 billion Euros and the cost of acquiring the drugs alone was around nine billion Euros (Sobocki et al., 2006). It was estimated that the cost of selective serotonin reuptake inhibitor treatment far exceeds ten billion US Dollars per year worldwide (Nestler et al., 2002).

Depression does not discriminate and it affects people of all ages, race and economical classes and influences virtually all aspects of existence. This includes the individual's psychological, social, mental and even biological wellbeing, resulting in alterations in both personal and professional spheres of life.

It has been estimated by the World Health Organisation that approximately 121 million people worldwide are affected by MDD and that it is the fourth most important cause of loss in disability adjusted life years worldwide (Kiss, 2008; Longone et al., 2008; Rex et al., 2004). In the age group 15-44 years it is predicted that MDD will become the second leading cause of disability (both genders combined) by 2020 (World Health Organisation, 2011a). The World Health Organisation (WHO) has estimated that about 877,000 persons die from suicide each year. They also reported that attempted suicides are 20 times more frequent than completed suicides and that mental disorders such as MDD, are associated with more than 90% of all cases of suicide (World Health Organisation, 2011b).

It is disturbing that only one third of patients treated with a single antidepressant achieve total remission (Trivedi et al., 2006) and that about one third of patients remain unresponsive to multiple treatment strategies. Furthermore, it is known that MDD is not limited to adults only and can affect individuals of very young ages. The impact that the disease might have on children and adolescents has been investigated in a number of studies. However, the long-term effects of such treatments on neurodevelopment and long-term psychological outcome still remain unclear.

### **2.1.1 Epidemiology**

MDD is a debilitating and serious mental illness that affects an estimated 2–5% of the population worldwide and has a lifetime prevalence of around 15% (Bylund & Reed, 2007). When comparing statistics, there is clear demographic variation across the globe. In the United States of America it is estimated that MDD affects between 4.1% and 10% of the population each year (Kessler et al., 1993; Waraich et al., 2004). Data published by Tomlinson et al. suggests that there is a lifetime prevalence of 9.7% in the Republic of South Africa (RSA) for a Major Depressive Episode (MDE) and this is higher than the prevalence for any mood disorder. However in the USA the lifetime prevalence is 21.4% which is significantly higher (Tomlinson et al., 2009). The prevalence can be defined as the total number of case of a disease in a given population at a specific time. It is important to note that there is a marked difference between the amount of affected people and the prevalence of the disease.

When comparing the prevalence of MDD to other mental disorders it is found to be much lower than the heritability of bipolar disorder or schizophrenia and is estimated to be in the region of 31-42%. This is most likely on the lower range and can be expected to increase with more reliable diagnosis of MDD (Kendler, 1983). MDD is caused by multiple genes and does not follow Mendel's laws of inheritance. It is a result of a complex interplay of genes and environmental risk factors and other common multifactorial diseases (Kessler, 1997).

It is suggested that women are more likely to suffer from MDD than men and research suggests that the incidence is almost twice of that in men (Earls, 1987). According to the National Comorbidity Study of the USA the prevalence of MDD in men is 12.7%, whereas in woman it has been estimated to vary between 17 and 21.3% (Blazer et al., 1994; Ververs et al., 2006).

#### **2.1.1.1 Major depressive disorder in children and adolescents**

Even though until the early 1970's depression was ignored in children and adolescents (Malkesman & Weller, 2009), it is now recognised that MDD may indeed present in these young patients. Some data suggest that the number

of incidents in this population group is on the rise and it is estimated that MDD affects 2.8% of children under the age of 13 years and 5.6% of adolescents older than 14 years but younger than 18 years (Jane Costello et al., 2006). It has also been estimated that almost 25% of children will have experienced a MDE before reaching adulthood (Kessler et al., 2001).

Even though depression does not follow Mendel's law of inheritance, data suggest a strong heritability factor for MDD. If a first degree relative suffers from MDD, the child has up to a 42% risk in developing the same condition (Sullivan et al., 2000). This percentage increases to 60% risk of developing MDD or a related psychiatric disorder if the child belongs to a family with two or more generations affected by MDD (Weissman et al., 2005).

The increase in the use of antidepressants in children represents one of the fastest growing treatments in the psychiatric community (Zito et al., 2000; Zito & Safer, 2001). In the USA, prescription rates for fluoxetine in preschool children as well as children in elementary school have increased 1.8-fold between 1991 and 1995 (Zito et al., 2000). In Canada the outlook is much worse, where a study showed a 10-fold increase between 1993 and 1997 in children five years and younger (Minde, 1998).

#### **2.1.1.2 Major depressive disorder in pregnant and lactating women**

Developing children can be exposed to antidepressants in several ways. This include foetuses who may be subjected to placental transfer of antidepressant drugs from the mother, or new-born babies who are exposed via the excretion of the drug through breast milk (Kinney et al., 2007). The latter examples highlight the importance to understand the effect of these drugs on the foetus and new-born babies.

In their childbearing years women are at the highest risk for developing depression (Blazer et al., 1994) and this risk ranges between 9 and 16% (Bennett et al., 2004; Evans et al., 2001; Josefsson et al., 2001; Oberlander et al., 2006). Add to this the data that shows that women are twice as likely to develop MDD when compared to men (Earls, 1987; World Health Organisation, 2011b), it increases the likelihood that pregnant women may be

taking an antidepressant. This includes therapy initiated prior to the pregnancy, or initiation of therapy during pregnancy (Field, 2010; Gentile & Galbally, 2011; Nonacs et al., 2005; Ververs et al., 2006).

It has been estimated that 0.5% of women will start using an antidepressant during pregnancy (Ververs et al., 2006), and that up to 25% of depressed women that are already on antidepressant treatment, will continue the therapy during pregnancy. This occurs because discontinuation of treatment with the antidepressant during pregnancy gives a significant increase in the frequency of relapse of depression in the mother (Cohen et al., 2006). The drug of choice for treatment of depressed pregnant women is fluoxetine (Nonacs & Cohen, 2003). Its use and that of other SSRIs has showed a marked increase in the last two decades in this treatment group (Andrade et al., 2008; Cooper et al., 2007; Oberlander et al., 2006; Vaswani et al., 2003; Ververs et al., 2006). However, safety is not well established and in all of these instances it is important to consider whether the benefit outweighs the risks involved. In particular, there is no certainty whether neurodevelopment could be altered or major foetal malformations could result in specific cases (Louik et al., 2007).

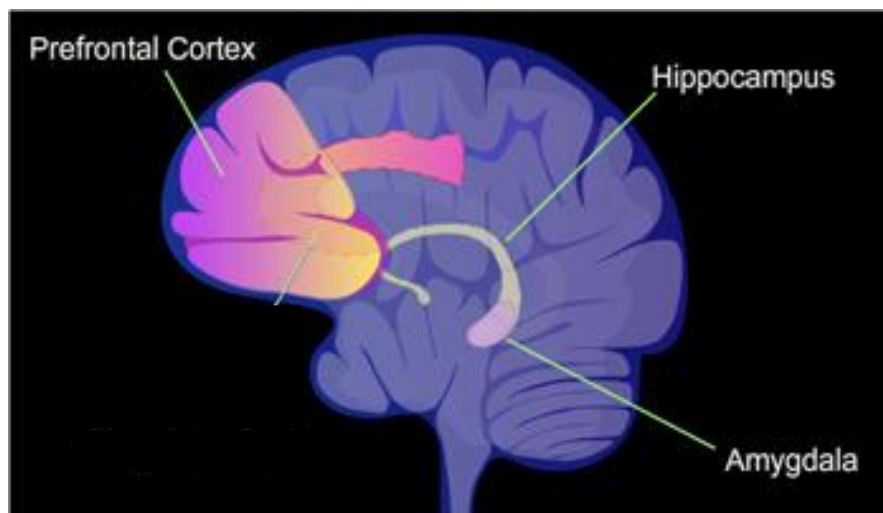
It has also been found that depression during pregnancy is associated with an increased risk of preterm delivery, decreased birth weight and higher number of admissions to the neonatal intensive care unit of new-born babies (Bonari et al., 2004; Chung et al., 2001; Field et al., 2010). The adverse effects of depression during pregnancy has been shown to affect neurodevelopment (such as developmental delay) (Deave et al., 2008), lowering IQ in adolescence (Hay et al., 2008) and impairing language development (Nulman et al., 2002; Paulson et al., 2009) in the offspring. It is believed that these adverse effects can be prevented by the effective treatment of depression in pregnant women, hence affecting the potential benefit-risk ratio.

### **2.1.2 Neurobiology (anatomy and neuropathology) of MDD**

A host of evidence suggest that major depressive disorder is a neurodegenerative disease and particularly in severe cases of resistant MDD, it is associated with functional and even structural changes of the prefrontal cortex, cingulate cortex, hippocampus and amygdala (Drevets et al., 2008)

(see Figure 2-1). Evidence suggests that specific connections between these areas are lost (Mayberg, 2003) and animal studies suggest that the probable cause is the disappearance of spines on the neuronal dendrites. This disappearance of dendrites then leads to the regression of synapses (Ao, 2008).

The majority of the studies on the neuropathophysiology of depression have been performed in adults, but there are a limited number of neurobiological studies that suggest that the brain regions affected in children with depression are comparable to those in affected adults (Andersen & Navalta, 2004; Kowatch et al., 1999).



**Figure 2-1:** Illustration of the three major areas affected by MDD.

### **2.1.2.1 The prefrontal cortex**

The prefrontal cortex (PFC) is the mediator of key cognitive processes in the brain. The medial prefrontal cortex (MPFC) enables us to reflect on the values that other people attach to actions and outcomes as well as what other people think about us (Amodio & Frith, 2006).

Magnetic resonance imaging (MRI) indicated a 1.8% decrease in prefrontal cortical grey matter of patients with MDD (Koolschijn et al., 2009). However, there are no reports of changes in neuron dimensions or densities in the prefrontal cortex in MDD (Miguel-Hidalgo et al., 2000). On the other hand there is a reported decrease of up to 40% in markers for oligodendrocytes in

the grey matter of MDD patients in the anterior frontal cortex (Honer et al., 1999). These measurements suggest a loss of 40% of satellite oligodendrocytes and would account for a volume change of about 2.0% in the anterior frontal cortex (Bennett, 2013). It could also explain the observed 1.8% decrease in grey matter detected by MRI, if the grey matter was to be decreased because of this loss of satellite oligodendrocytes. However, no data indicated changes in markers for synaptic boutons, such as synaptophysin, in the anterior frontal cortex of patients with MDD (Honer et al., 1999).

The most fundamental and prolonged changes of the PFC (Figure 2-1) occurs during adolescence, especially when comparing it to the changes seen in regions such as the primary motor and sensory cortices (Bourgeois et al., 1994; Peter, 1979). These changes involve the pruning of the synapses (which causes a reduction in grey matter) and an increase in myelination (which is responsible for an increase in white matter) (Giedd et al., 1999).

It has been demonstrated in children suffering from depression that there are indeed changes in the PFC when compared to that in non-depressed controls. Depression in children causes changes such as increased frontal grey matter and decreased frontal white matter (Steingard et al., 2000). Studies have also found a reduction in regional cerebral blood flow (rCBF) in the left anterofrontal lobe of the brain (Tutus et al., 1998) and dysfunction of the frontal lobe as measured by electrophysiological readings (Steingard et al., 2000).

Importantly, these changes that occur in children and adolescents suffering from MDD are comparable to those seen in adults (Andersen & Navalta, 2004; Kowatch et al., 1999).

#### **2.1.2.2 The hippocampus**

Learning and verbal memory are some of the processes sustained by the hippocampus (Reiman, 1997) (Figure 2-1).

Numerous studies have demonstrated a smaller left hippocampal size in patients suffering from depression vs. healthy controls (Bremner et al., 2000; Frodl et al., 2003; MacMaster & Kusumakar, 2004; MacQueen et al., 2003). A

meta-analysis of MRI studies of patients with MDD indicates that the hippocampus can lose up to 5% of grey matter (Koolschijn et al., 2009). This decrease in volume can be explained by the reported loss of synapses in the hippocampus (Eastwood & Harrison, 2000), accounting for less than 1% of the loss, and a concomitant loss of dendrites that accounts for up to 4.8% of the loss (Bennett, 2013). It should be noted that these figures are conditional on the presence of similarities in the distribution of cells and their processes in the grey matter of the cortex and hippocampus. However, this is not the case because the length of pyramidal cell dendrites in the hippocampus is greater than those in the cortex. Unfortunately there are no observations to confirm the nature of these losses in the hippocampus in MDD. Also, it has not been clearly demonstrated to which extent these changes reflect in altered neuronal structure, neuronal body volume, synaptic sprouting, and total water, protein and lipid content.

Early studies have shown that hippocampal neurogenesis and plasticity are influenced by stress, as demonstrated in MDD (Reagan & McEwen, 1997; Woolley et al., 1990). Chronic stress, such as with MDD, has been associated with dendritic remodelling of the synaptic terminal structures (Sapolsky et al., 1985; Sapolsky et al., 1990; Uno et al., 1989) resulting in the death of cells in certain brain regions (Czéh & Lucassen, 2007; Harlan et al., 2005; Sousa & Almeida, 2002).

Young patients with a familial history of MDD present with a decreased hippocampal volume, suggesting that these individuals may be at a higher risk for developing MDD later in life (MacMaster et al., 2008). For example, a decrease in hippocampal volume has been demonstrated in adult male patients suffering a first-time MDD episode (Frodl et al., 2002). It seems as though gender plays an important role in the development of depression and this warrants further investigation.

In summary, there is a consensus that impaired hippocampal function and reduced volume is associated with MDD. The exact nature and clinical implications of such changes are not clearly understood.

### **2.1.2.3 The amygdala**

The amygdala is located deep within the anterior inferior temporal lobe and is important in emotional memory. It could therefore mediate anhedonia (decreased drive and reward for pleasurable activities), anxiety, and reduced motivation that are dominant in many patients that suffer from MDD (Nestler et al., 2002).

Neuroimaging, electrophysiological and lesion analysis studies both in humans and in experimental animals have demonstrated that the amygdala is involved in the recollection of emotional or arousing memories (Canli et al., 2000; LeDoux & Bemporad, 1997; Phelps & Anderson, 1997). In humans electrical stimulation of the amygdala can evoke emotional experiences (especially fear or anxiety) (Brothers, 1995; Cahill et al., 2001; Canli et al., 2000; Gloor et al., 1982) and recollection of emotionally charged life-events (Brothers, 1995). There is also elevated amygdala metabolism present in MDD. All of these observations suggest that excessive amygdalar stimulation of the cortical structures involved in declarative memory could be a reason why depressed subjects ponder on memories of emotionally unpleasant or guilt-provoking life events (Cahill et al., 2001).

Mood disorders that cause a dysfunction in the amygdala may also alter the initial evaluation and memory consolidation of sensory or social stimuli in regard to their emotional significance. The amygdala is also involved in the acquisition, consolidation and expression of emotional/arousing memories (Büchel et al., 1998; Canli et al., 2000; LaBar et al., 1998; LeDoux & Bemporad, 1997; Phelps & Anderson, 1997). It also plays a role in recognizing fear and sadness in facial expressions (Adolphs et al., 1994; Blair et al., 1999; Morris et al., 1996) and fear and anger in spoken language (Scott et al., 1997). Norepinephrine (I-NE) released in the amygdala plays a critical role in certain types of emotional learning (Cole & Robbins, 1987; Ferry et al., 1999; Rasmussen et al., 1986). The activation of NE neurons is facilitated by the effect of glucocorticoid secretion (Ferry et al., 1999). People suffering from depression have an abnormally elevated secretion of both I-NE and cortisol (Musselman & Nemeroff, 1993; Veith et al., 1994; Wong et al., 2000). The

aforementioned increase may enhance the likelihood that ordinary social or sensory stimuli are recognised or remembered as being aversive or emotionally arousing (Drevets, 2001).

In several studies a change in the size of the amygdala have been observed in patients with affective disorders (Altshuler et al., 1998; Altshuler et al., 2000; Karjalainen & Lehtonen, 2000; Sheline et al., 1998; Strakowski et al., 1999; Tebartz van Elst et al., 2000). Currently there is no data to support a significant difference in the volume of the amygdala of patients with recurrent depressive episodes (when compared to healthy controls), but studies have shown that patients with a first major depressive episode present with an increased amygdala volume when compared to patients with recurrent MDEs (Frodl et al., 2003; Karjalainen & Lehtonen, 2000; Sheline et al., 1998).

The amygdala is a highly plastic brain structure in which new cells are continually generated into adulthood (Carrillo et al., 2007; Keilhoff et al., 2005). Nevertheless, prenatal stress has been associated with a reduced density of proliferating cells in the amygdala in the developing brain (Kawamura et al., 2006), which can cause an increased risk for the development of psychiatric disorders.

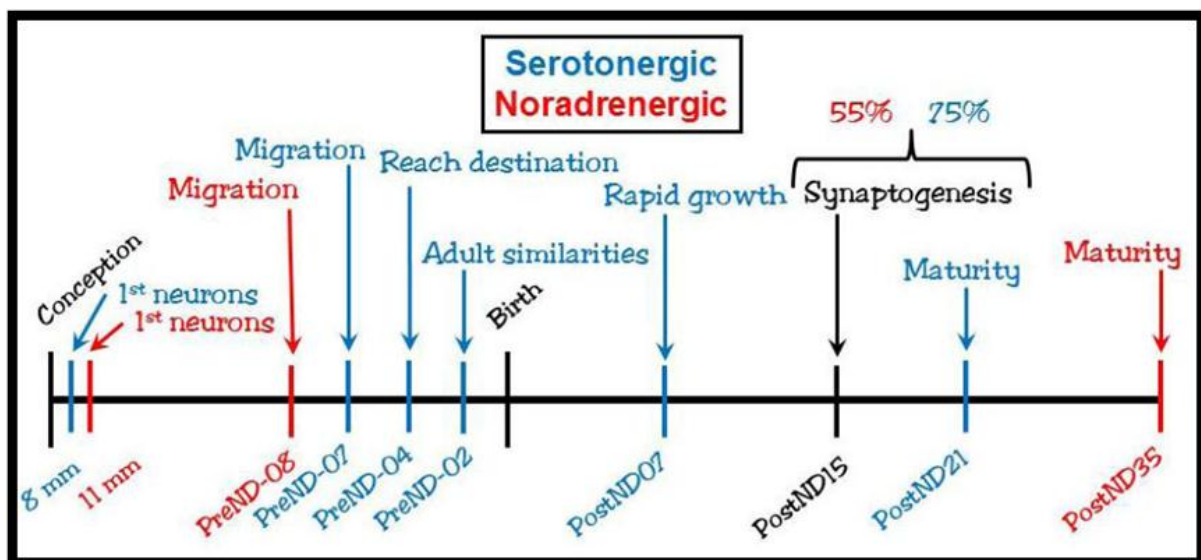
### **2.1.3 Neurotransmitter pathways involved in neurodevelopment**

There is a big difference in the rates of brain development across different species. However, the general age-related pattern of neuronal maturation, when comparing several neurobiological parameters, remains similar across most mammalian species. Neuronal maturation and brain development have been studied in various mammalian species, with the most data available for rodents. These findings are very important for the current study, and for this reason brain development in the rat will be discussed in more detail.

It is not easy to compare the development of the human brain to similar development in the rat brain and it is important to take several important factors into account. One of the most important factors is that it has been demonstrated that at birth the weight of the rat brain is comparable to that of the human brain in the second trimester. Moreover, rats reach sexual maturity

at about five weeks of age which corresponds to adolescence in humans (Murrin et al., 2007; Zeinoaldini, 2005). These hormonal changes markedly affect brain development and adolescence is seen as an important marker for certain hallmarks in brain neurobiological development. It is therefore important to keep these relative age-related differences in mind when interpreting neurodevelopmental data (Steyn, 2011).

A time-line demonstrating the relationship between age and serotonergic or adrenergic development, respectively, is depicted in Figure 2-2 (Steyn, 2011). Changes in the serotonin and norepinephrine content of specific brain regions of the rat embryo during pregnancy were investigated by Murrin and associates (Murrin et al., 2007). They demonstrated that serotonin-containing neurons are already present in the 8 mm sized rat embryo. In contrast norepinephrine-containing neurons were only observed at a later stage in development in the 11 mm rat embryo.



**Figure 2-2:** Timeline illustration of serotonergic and noradrenergic pathway development in rodents. (Steyn, 2011)

The blue sections of Figure 2-2 show that serotonergic neurons in the rat start projections to adult-like pathways by ND-07 and reach their destination by ND-04 (Wallace & Lauder, 1983). Serotonergic pathways already show strong similarities to adulthood two days before birth. However, at this time no

significant changes are visible in the noradrenergic system (Aitken & Törk, 1988; Wallace & Lauder, 1983).

There is a rapid increase in serotonergic neurons in the rat around seven days after birth (Figure 2-2), increasing to levels that surpass that seen in adult rats (Murrin et al., 2007). This rapid increase shows a distinct relationship to the increase in serotonin-labelled varicosities that increase by 20% compared to numbers present at birth (Dinopoulos et al., 1997). Normal levels as seen in adult rats will be reached around ND+21 (Figure 2-2) as levels will decrease to normal levels after the initial increase in neurodevelopment (Andersen & Navalta, 2004).

The red sections of Figure 2-2 shows that the development of noradrenergic neurons is different to that of serotonergic neurons in that the migration of cortical noradrenergic neurons initiates around day ND-08 and continues throughout early postnatal development.

Synaptogenesis of the serotonin pathway reaches approximately 75% of adult levels in the raphe nucleus of the rat brain by ND+15. Lagging behind in development, the norepinephrine synaptogenesis is only at 55% of adult levels in the locus coeruleus the brain at ND+15 (Figure 2-2) (Lauder & Bloom, 1975). By ND+15 serotonin 5-HT<sub>7</sub> receptor types are expressed, but they are almost completely absent by ND+21 (Vizueté et al., 1997), believed to be the result of “synaptic pruning”. The sprouting of cellular processes and the formation of synaptic contacts with neighbouring neurons is called maturation, with maturation of cortical neurons occurring mainly within the first three weeks of postnatal development. This correlates with the time in neurodevelopment when noradrenergic interventions increase to adult levels (Berger-Sweeney & Hohmann, 1997; Markus & Petit, 1987).

As seen in Figure 2-2 the serotonergic system reaches maturity by ND+21, whereas the noradrenergic system only reaches maturity by ND+35 and continues to develop throughout postnatal development (Murrin et al., 2007).

It is clear from the above that the most fundamental development of serotonin pathways occurs mainly during the prenatal developmental phase, culminating

in full maturation in the first few weeks after birth. In contrast, the noradrenergic system only reaches final maturation weeks after the serotonin system and mainly develops postnatally (Murrin et al., 2007).

The reasoning for using rats in this study is its similarity in neurodevelopmental timeline compared to the human brain. For example, at around five to six weeks of development, the norepinephrine neurotransmitter is already detectable in the human foetus. The levels of norepinephrine at this time also correlate with that seen in the rat. I-NE levels increase throughout the first trimester, especially from two months of gestation, after which there is a decrease of 30-40% between the ages of six months and early childhood (Murrin et al., 2007). Also, it has been found that the 5-HT pathway develops earlier than the NE pathway in humans and also reaches maturity by adolescence, corresponding to the development seen in the rats (Andersen & Navalta, 2004).

#### **2.1.4 Hypotheses of major depressive disorder**

Both environmental factors such as stress along with neurobiological susceptibility play a role in the development and manifestation of MDD (Kendler & Karkowski-Shuman, 1997). In this regard, there is a significant evidence base supporting a neurobiological basis for MDD and several hypotheses in this regard have been postulated and/or refined over the past decades. These hypotheses each emphasise a key neurobiological characteristic of MDD and the debate is continuing to establish the most unifying hypothesis (Leonard, 2004). This section will discuss the most relevant hypotheses to the current study.

##### **2.1.4.1 The monoamine hypothesis**

The monoamine hypothesis of depression emerged from various clinical and experimental observations. Firstly, reserpine an irreversible monoamine storage depleter has been shown to induce depression in certain individuals (Sapolsky, 2000). Secondly, drugs that inhibit the reuptake of norepinephrine (tricyclic antidepressants) or that inhibit the breakdown of norepinephrine (monoamine oxidase inhibitors) alleviate depressive symptoms (Schildkraut, 1965). The original monoamine hypothesis therefore proposed that affective

disorders are caused by a shortage of catecholamines (Coppen, 1967; Schildkraut, 1965) and/or indoleamines (Coppen, 1967) at important receptor sites in the CNS. The therapeutic effects of antidepressants were then postulated to result from increased stimulation of norepinephrine and/or serotonin receptors, specifically by increasing the brain concentrations of these monoaminergic neurotransmitters. However, it was soon demonstrated that this hypothesis is too simplistic and it has been refined several times since.

Several studies have been performed to assess the validity of the monoamine hypothesis. One such study evaluated the effects of diet-induced monoamine depletion on depressive symptoms in depressed patients and healthy controls. The data demonstrated that class of antidepressant used as well as the type of diet-induced monoamine depletion affected the probability of relapse (Delgado et al., 1999). Antidepressants or diets affecting the same monoaminergic system resulted in similar probabilities of relapse. Depletion of serotonin, norepinephrine or dopamine did not decrease mood in healthy controls and only slightly lowered mood in healthy controls with a family history of MDD (Delgado et al., 1999). These depletion studies failed to demonstrate a relationship between serotonin and norepinephrine with depressive disorder (Cowen, 2008; Mendelsohn et al., 2009; Ruhé et al., 2007). After these findings were published, a revised monoamine theory of depression was formulated (aan het Rot et al., 2009; Heninger et al., 1996), stating that monoamine systems are only modulating other brain neurobiological systems that have a primary role in depression.

The mitochondrial enzyme, monoamine oxidase (MAO), regulates metabolic degradation of the catecholamines and serotonin and forms part of the regulation of monoamine neurotransmission and of reactive oxygen species (ROS) production. Membrane transporters for monoamine neurotransmitters determine extracellular concentrations of the neurotransmitter and strongly regulate signal transduction at synapses. The serotonin transporter (SERT) is a target of many antidepressants and is the focus of much interest. It has been suggested that persons with reduced expression of SERT are more

sensitive to stress-induced depression (Caspi et al., 2003). The modified monoamine theory (Meyer et al., 2006) suggests that serotonin or norepinephrine levels in the brain are mainly regulated by MAO type A (MAO-A) activity. It also suggests that the severity of symptoms of depression is directly related to changes in specific brain areas in the activity of monoamine transporters. This implies that an increase in MAO-A activity and the modified density of transporters are part of the pathophysiology of affective disorders. This hypothesis (the advanced monoamine hypothesis) was supported by observation that during a major depressive episode MAO-A density was elevated, resulting in greater metabolism of monoamines in the brain (Meyer et al., 2006; Meyer et al., 2009), and a decreased SERT binding in the brain (Selvaraj et al., 2011).

In summary, several studies over many years support a role for monoaminergic neurotransmission in the pathophysiology of depression, although its role has not been demonstrated to be conditional for the development of the disorder. It is now realised that the mechanism of action of antidepressants does not necessarily have to be opposite of what is observed in the pathophysiology of depression (Krishnan & Nestler, 2010), eg. the antidepressant and 5-HT reuptake enhancer, tianeptine. Direct measurements of monoamine concentrations do not always yield definitive evidence for a direct relationship between monoamine concentrations and antidepressant response, so that the downstream effects of monoamine neurotransmission needs to be explored to better explain antidepressant action (Belmaker & Agam, 2008), including that of the SSRIs, SNRIs and TCAs.

#### **2.1.4.2 The cholinergic super-sensitivity hypothesis**

In the early 1970s, Janowsky and colleagues first introduced the cholinergic super-sensitivity hypothesis of depression (Janowsky et al., 1972). It postulates that hyper- and hypo-cholinergic states are associated with depression and mania, respectively. The hyper- and hypo-cholinergic states then acts to strengthen complementary decreased and increased noradrenergic neurotransmission (Dilsaver, 1986; Janowsky et al., 1972).

The hypothesis was originally based on the observation that organophosphate poisoning in humans led to depressive-like symptoms. Organophosphates inhibit the enzyme acetylcholinesterase (AChE) which results in increased levels of acetylcholine (ACh) throughout the brain and periphery (Gershon & Shaw, 1961). Central acting AChE inhibitors (such as physostigmine), but not peripheral active inhibitors (such as neostigmine) has been shown to induce depressive-like symptoms in human patients (Janowsky et al., 1974).

The cholinergic-super-sensitivity hypothesis of depression has been limited by a lack of antidepressant activity by some, but not all anticholinergic drugs. Pro- and antidepressant effects are controlled by a small balance between the activation and desensitization of cholinergic receptors, so that the cholinergic system is mostly viewed as a contributor to mood and not as a critical determinant (Picciotto et al., 2008). Indeed, animal studies have demonstrated its important regulatory function in antidepressant response (Brink et al, 2008; Liebenberg et al, 2010), while anticholinergics have been found to increase antidepressant response in treatment refractory depressed patients (Manji et al., 2001). Due to its limitations and the fact that no clinically used antidepressant is known to evoke its effect by any significant contribution of anticholinergic action, this hypothesis has not received a great deal of attention through the years and has as a result been overshadowed by other hypotheses.

#### **2.1.4.3 The hypothalamic-pituitary-adrenal-axis hypothesis**

The hypothalamic-pituitary-adrenal-axis (HPA-axis) hypothesis states that chronic elevated levels of corticosteroids lead to decreased neurogenesis and manifests as MDD (Höschl & Hajek, 2001; Mizoguchi et al., 2003; Sheline et al., 1996; Sheline et al., 1998). The body's response to acute or chronic stress is mediated by the HPA-axis. In reaction to stress there is a release of corticotrophin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus. This is a stimulating hormone that causes the secretion of adrenocorticotrophin (ACTH) from the anterior pituitary. The ACTH is then in turn responsible for the stimulation of the adrenal glands and the release of glucocorticoids (cortisol in humans and corticosterone in rodents). The

glucocorticoids have an extensive effect on the immune system, behaviour, general metabolism and certain brain functions (Ehlert et al., 2001; Herbert et al., 2006; Schimmer & Parker, 1996). The hormonal pathway uses negative feedback inhibition to suppress the cascade in the hypothalamus and to prevent further release of cortisol from the adrenal glands.

There is significant evidence in the more severe forms of MDD that there is an enhanced activity of the HPA axis. Abnormal and excessive HPA activation is observed in approximately half of individuals suffering from MDD and these abnormalities are corrected by antidepressant treatment (Arborelius et al., 1999; De Kloet et al., 1988; Holsboer, 2001; Sachar & Baron, 1979). The enhancement in HPA activity has been connected to an increased adrenal gland volume, a greater frequency of cortisol release and marked reductions in bone mineral density, as well as increased risk of diabetes, obesity and metabolic syndrome when compared to healthy controls. There is also evidence that corticosteroid receptor function is reduced in many patients suffering from MDD, as well as in healthy individuals that have an increased genetic risk for a depressive disorder (Holsboer, 1999).

#### **2.1.4.4 Neurotrophic, Neuroplasticity and Neurogenesis Hypotheses**

A more unifying hypothesis of the neurobiological basis of depression resulted from the analysis of complex subcellular processes underlying neuroplasticity. In particular, our association of neuroplasticity with depression was prompted by a lack of understanding of the mechanism of action of mood stabilisers (eg. lithium that does not exert overt effects on monoaminergic systems) and other newly introduced antidepressants without direct monoaminergic actions, such as agomelatine. In particular, existing alternative hypotheses have been unable to explain their mechanisms of action.

The neurotrophic hypothesis of depression (Duman et al., 1997; Duman, 2002; Einat & Manji, 2006; Zarate Jr et al., 2006) states that there is a deficiency in neurotrophic factors and this may contribute to hippocampal pathology in the development of depression. The reversal of this deficiency, either by antidepressant treatment or the use of mood stabilizers, may contribute to the resolution of depressive symptoms. The hypothesis

postulates that vulnerability to depression can occur as a result of neuronal damage brought about by a deficiency in neurotrophic factors and/or an excess of neurodamaging factors such as glutamate. Thus, the therapeutic effects of antidepressants may consist of increased function of the monoaminergic system and this increase in the monoaminergic system leads to higher expression of the neurotrophin, brain derived neurotrophic factor (BDNF) and its receptor TrkB. The increase in BDNF subsequently leads to an increase in neuronal plasticity and the normal resumption of cellular functions after stress (Carlezon Jr et al., 2005). It has been suggested that the effects of antidepressant treatments could be explained by the reactivation of BDNF-mediated cortical plasticity. This will then lead to an adjustment of the neuronal networks to better adjust to environmental challenges (Castrén & Rantamäki, 2010).

The neurogenesis hypothesis of depression proposes that depression is caused by impaired hippocampal neurogenesis while antidepressant treatment is effective by stimulating neurogenesis in this brain region (Sapolsky, 2004). Confirmation that reduced hippocampal neurogenesis is part of the pathophysiology of depression requires further research (Gass & Riva, 2007; Santarelli et al., 2003).

In summary, it is proposed that neurodegeneration or reduced neurogenesis may be an important mechanism to explain depression. Neurotrophins are also seen as key regulators of neurogenesis and neuroplasticity (Pittenger & Duman, 2007). An increase in neuroplasticity increases the recovery of brain networks and this induces an antidepressant effect. It is thought that structural and functional brain abnormalities in patients with MDD could be associated with low levels of BDNF as well as an abnormal functioning of the HPA axis and glutamatergic toxicity (aan het Rot et al., 2009; Krishnan & Nestler, 2008; Mathew et al., 2008).

Early findings made in the early 1990's showed that N-methyl-d- aspartate receptor antagonists possess antidepressant-like action and these findings can be seen as the origin of the glutamate hypothesis (Trullas & Skolnick, 1990). In recent times changes have been made to the hypothesis to include

intracellular signalling, neurogenesis, neurotrophic mechanisms and synaptic plasticity and now falls under the neuroplasticity hypothesis (Pittenger & Duman, 2007; Racagni & Popoli, 2008; Sanacora et al., 2008). It is known that a vast majority of brain neurons and synapses are glutamatergic, and that glutamatergic synaptic transmission mediates both cognition and emotion in the brain (Pessoa, 2008).

### **2.1.5 Diagnosis**

Diagnosis of MDD is done according to a set of subjective measures as determined by a clinician after a contact session with the patient. An episode of MDD is diagnosed in an adult patient according to the Diagnostic and Statistical Manual of Mental Disorders 4th ed. (DSM-IV), when one of the first two symptoms, plus any other four, listed below (Table 2-1), presents for at least two weeks and disrupts the normal daily functioning of the individual (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV., 1994). The criteria for the diagnosis of MDD in children and/or adolescents are the same as those used for adults (Son & Kirchner, 2000).

**Table 2-1:** Diagnostic criteria for the diagnosis of MDD according to the DSM-IV (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV., 1994).

- 
- Depressed mood, but can present as irritable mood in children and adolescents
- 
- Decreased interest or pleasure in most activities, most of each day
  - Significant weight change (5%) or change in appetite
- 
- Changes in sleep patterns such as insomnia or hypersomnia
  - Changes in activity such as psychomotor agitation or retardation
- 
- Fatigue or loss of energy
  - Feelings of worthlessness or excessive or inappropriate guilt
- 
- Diminished ability to think or concentrate, or more indecisiveness
  - Thoughts of death or suicide, or has suicide plan
- 

One of the problems with the diagnosis of MDD is that it relies on a set of variable and relative subjective systems and not on an objective diagnostic test, making diagnosis relatively specialised. Major depression may be viewed as a heterogeneous syndrome, presenting with varying patterns of a number of distinctive symptoms (Liebenberg, 2009).

### **2.1.6 Signs and symptoms**

The clinical presentation of MDD is the same for children, adolescents and adults (Kovacs, 1996).

Symptoms that are associated with MDD include mood, behavioural, cognitive, psychomotor and other related dysfunctions, such as the loss of social, cognitive and interpersonal skills and interest, social withdrawal, poor

school attendance, impaired or irritable family and peer relationships, feeling “blue” or tired, depressed mood, and presenting with a decreased appetite. The risk for self-harm, suicide ideation and thoughts of death are increased in these patients. Childhood depression may also promote personality disorders in susceptible individuals, since the depression interferes with the developing personality (Andersen & Navalta, 2004; Bylund & Reed, 2007; Weissman et al., 1999). It has been suggested that clinical presentation in children could be different to that in adults. Children are more likely to present with somatic symptoms, restlessness, separation anxiety, phobias and hallucinations. Adolescents on the other hand are more likely to experience anhedonia, boredom, hopelessness, hypersomnia, weight change, alcohol or drug abuse and suicide attempts (Soutullo & Figueroa-Quintana, 2013; Williams et al., 2009). The signs and symptoms are the most difficult to observe in infants and pre-schoolers, as they do not have the ability to express feelings of sadness in language. In this age group, depressive symptoms must be inferred from gross changes in behaviour, including apathy, withdrawal from caregivers, delay or regression of developmental milestones and failure to thrive without any evidence of a physiological/organic cause (Son & Kirchner, 2000).

### **2.1.7 Treatment options for major depressive disorder**

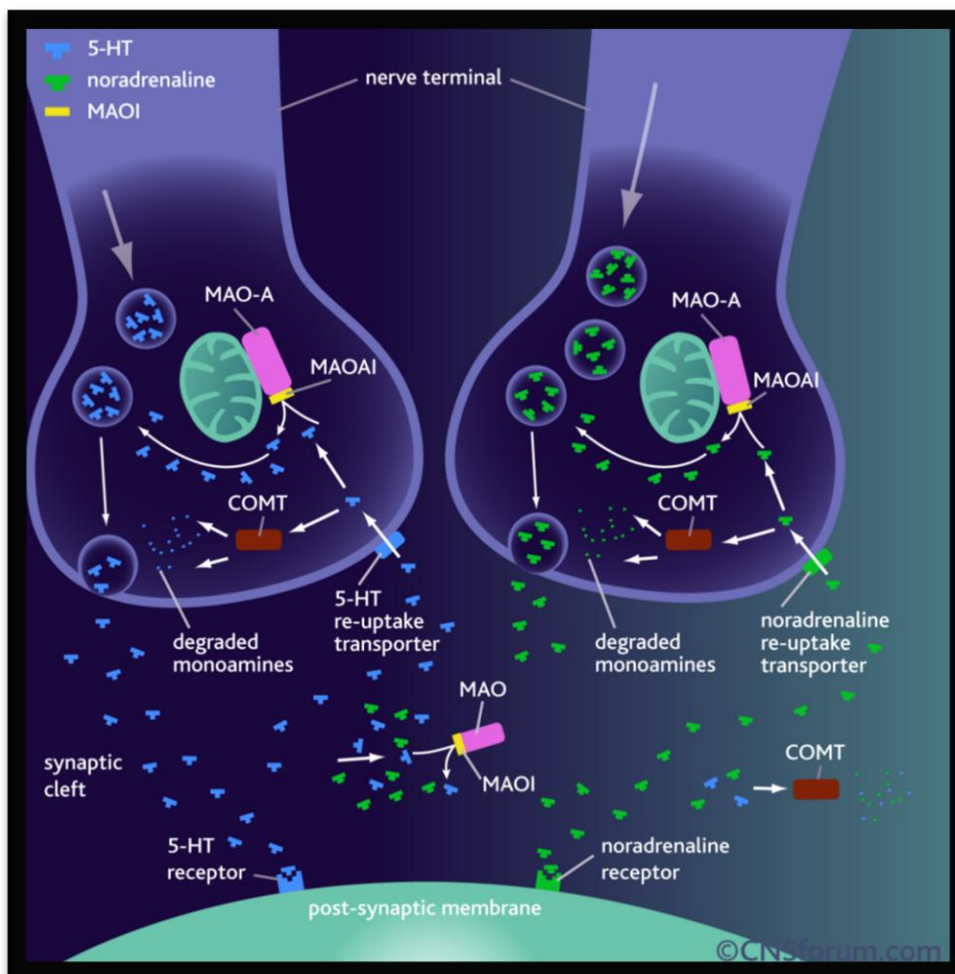
There are five main classes of antidepressants that are used clinically to treat MDD. They are usually classified according to their neurobiological mechanism of action. Treatment resistant depression has been a strong driving force for identifying novel neurobiological targets, and hence for the development of new antidepressants to treat the disorder. Each of the different classes of antidepressants currently available is discussed below, with reference to their mechanisms of action.

When initiating treatment of MDD, the clinician should exclude any underlying causative medical condition, drug therapy, or substance abuse. Where possible, such underlying causes of secondary depression should be addressed first, and in some instances antidepressant treatment may be

needed together with the treatment of the underlying disease (Ciraulo et al., 2011).

When antidepressant treatment is indicated for first episodes of unipolar major depression, it is advised to continue treatment for at least 1 year. In cases of recurrent depressions, severe single episodes, onset of the first episode before the age of 20 years, and a family history of depression, antidepressant therapy may continue indefinitely (Ciraulo et al., 2011).

Most of the treatment options available today targets the neurotransmission of monoamines, their transport systems or their routes of metabolism. Figure 2-3 depicts the synaptic cleft with the different targets that are used in anti-depressive treatment.



**Figure 2-3:** Neurotransmission, transport systems and metabolism of monoamines in the synaptic cleft

### **2.1.7.1 The monoamine oxidase inhibitors**

Monoamine oxidase inhibitors (MAOIs) were the first antidepressants used in clinical practice. Iproniazid, a derivative of isoniazid, was originally developed by Herbert Fox at Roche Laboratories in 1951 for the treatment of tuberculosis (Fox & Gibas, 1953). It was found to be ineffective for tuberculosis, but it was noted to have a mood elevating effect in some of patients (Selikoff et al., 1952). It is believed that its antidepressant properties result from the inhibition of monoamine oxidase (MAO). MAO catalyses the oxidative deamination and deactivation of monoamines (dopamine, epinephrine, norepinephrine and serotonin) (Ban, 2001; Kline, 1958; Loomer et al., 1957; Zeller et al., 1952). The inhibition of MAO results in an increased availability of these biogenic amines in the synaptic cleft by preventing their metabolism resulting in an increased stimulus effect-response chain (Figure 2-3). Even though it has an extensive warning list and side effect profile, the pharmacological action of MAOIs are unique and should still be considered as alternative treatment when other antidepressants are not effective.

In the 1950s and 1960s the primary treatment option for depression was MAOIs. As clinical experience grew, the serious adverse effects of MAOIs became evident and iproniazid and pheniprazine were withdrawn from the market due to their hepatotoxicity (Ban, 2001). The introduction of safer antidepressants from different classes led to a steady decline in MAOI use and at this moment there are only four MAOIs approved by the FDA for treatment of depression in the United States. They are phenelzine, tranylcypromine, isocarboxazide, and selegiline (Papakostas, 2006). Phenelzine, tranylcypromine and isocarboxide are all MAO-A and MAO-B inhibitors, whereas selegiline is a selective inhibitor of MAO-B at low doses (Ciraulo et al., 2011). This is of importance because monoamine oxidase also metabolises tyramine and if tyramine is taken with a MAOI it could lead to serotonin syndrome. MAO-B selective inhibitors have a smaller chance of causing serotonin syndrome because tyramine can still be metabolised by MAO-A. Reversible inhibitors of monoamine oxidase, like moclobemide, also do not cause the reaction with tyramine because the tyramine can displace the inhibitor from the enzyme (Ciraulo et al., 2011).

Due to their potential interactions MAOIs are now considered third or fourth-line agents in the treatment of depression. The MAOIs have established efficacy in the treatment of atypical depression, bipolar depression, and dysthymia and it has been suggested by several studies to be superior to other established antidepressants in the treatment of depression (Guelfi et al., 1992; Liebowitz et al., 1988; Ocepek-Welikson & Rabkin, 1993; Van Vliet et al., 1993). MAOIs have been found to be extremely effective in the treatment of elderly patients with depression (Georgotas et al., 1986).

In a study done by Quitkin et al it was found that MAOIs were as effective as the tricyclic antidepressants (TCAs) in the treatment of depressed patients (Quitkin et al., 1990). It has been suggested though that higher than usual doses of MAOI may be needed in severely depressed patients and those who failed treatment with a TCA (Thase et al., 1995). The increase in dosage also means an increase in the risk associated with the side effects. Support for the use of MAOIs in patients who failed trials with other antidepressants is well documented and is a viable alternative treatment option for treatment resistant depression (McGrath et al., 1992; Thase et al., 1995; Tomlinson et al., 2009). MAOIs are also effective in dysthymia, anxiety, and phobic disorders (Vallejo et al., 1987) and there are also some reports of efficacy in PTSD and personality disorders, although some of the available data are contradictory (Cornelius et al., 1993; Davidson, 1997; Liebowitz et al., 1992; Stein, 1992).

#### **2.1.7.2 The tricyclic antidepressants**

The first antidepressant from the tricyclic antidepressant (TCA) class to be used in clinical practice was imipramine. Trials were done to test its efficacy as a potential anti-histamine and antipsychotic (Ban, 2001; Kuhn, 1958), but in 1957 its efficacy in the treatment of depression was reported by Roland Kuhn (Kuhn, 1958). A number of years later, an analysis of 23 published studies by Klerman and Cole demonstrated the superiority of imipramine to placebo in depressed patients. It was found that 65% of patients from those studies improved clinically on imipramine compared to only 31% improvement among placebo-treated patients (Klerman & Cole, 1965).

The TCAs were the first-line agents for the treatment of depression for over 30 years and are still used today. During this time it was found that imipramine also has an active metabolite (desipramine, the demethylated metabolite of imipramine). In the 1960's a new TCA was introduced in the guise of amitriptyline and later its metabolite nortriptyline was also marketed (Ciraulo et al., 2011). Over the past 20 years there has been an introduction of heterocyclic and other types of antidepressants, but none have demonstrated superior efficacy to the TCAs.

The TCAs offer advantages over MAOIs because they have a smaller risk of drug–drug interactions and they require no food restrictions. However they have troublesome adverse effects in many patients that include dry mouth, nausea, constipation or diarrhoea, headache, drowsiness, insomnia, dizziness and weight gain. They also have a small therapeutic index, especially cardio toxicity that can present problems in patients with suicidal risk. Currently, the following tricyclic and heterocyclic compounds are FDA approved for the treatment of depression in the United States: amitriptyline, amoxapine, clomipramine, desipramine, doxepin, imipramine, maprotiline, nortriptyline, protriptyline, and trimipramine (Ciraulo et al., 2011).

The antidepressant action of TCAs is believed to be due to the inhibition of norepinephrine (NE) reuptake and serotonin (5-HT) reuptake (Figure 2-3). This inhibition leads to increased concentration of these monoamines in the synaptic cleft leading to an increase in the stimulus effect-response chain. Subsequent down-regulation of postsynaptic receptors followed by changes in gene expression is believed to be ultimately responsible for their antidepressant action (Crews et al., 1983), although this has been attributed to most antidepressants and is linked to the neuroplasticity hypothesis described earlier.

Two subclasses of TCAs are notable, namely secondary and tertiary amines. These subclasses differ with regard to their spectrum of selectivity for reuptake transporters and activity on various specific receptors. These differences include that tertiary amine TCAs will inhibit the reuptake of both NE and 5-HT (Figure 2-3) while secondary amine TCAs preferably inhibits the

reuptake of I-NE (Figure 2-3) (Brunton et al., 2010c). Nortriptyline, amitriptyline, and clomipramine are also antagonists at the 5-HT<sub>2A</sub> receptor, although the clinical significance of this effect is still unknown. TCAs are also multi-potent and are known to have effects on  $\alpha_1$ , H<sub>1</sub> and acetylcholine receptors (O'Donnell & Shelton, 2011). This multi-potency is the cause of the extensive adverse effect profile of this class of antidepressants (Wijeratne & Sachdev, 2008).

### **2.1.7.3 The selective serotonin reuptake inhibitors**

Fluoxetine was the first selective serotonin reuptake inhibitor (SSRI) to be introduced to the American market in 1988 (DeVane, 1998). Other SSRIs such as sertraline, paroxetine, and fluvoxamine, followed shortly. Even though it was widely used in Europe for a number of years, it was not until the late 1990s that citalopram (another SSRI) became available on the American market. Its S-enantiomer, escitalopram followed some time later.

By the early 1990s, the SSRIs were seen as first-line antidepressants in clinical practice and accounted for more than 50% of all antidepressant prescriptions. The SSRIs enjoyed unparalleled marketing success (Ban, 2001), had greater exposure in literature and the news and were thought to be much superior to existing antidepressant drugs. The success of the SSRIs has since been ascribed not to superior therapeutic efficacy, but to their more favourable side effect profile, simpler dosing strategies and overall better tolerability, and consequently better adherence by the patients. The SSRIs are also relatively safe in overdose with minimal cardiovascular effects as well as lower anticholinergic activity, rendering them appealing as first-line agents. It has, however, become apparent that the SSRIs have their own share of adverse effects, especially those of a serotonergic nature such as headache, insomnia, drowsiness, nausea, sexual dysfunction, anorexia and tremor (Ciraulo et al., 2011). Their efficacy compared to the TCAs has also been questioned and remains a matter of controversy to this day. These concerns have not deterred the use of SSRIs and they are widely used and are effective in a wide range of psychiatric disorders other than depression. SSRIs are employed in the treatment of anxiety disorders, obsessive compulsive disorder

(OCD), panic disorder, bulimia nervosa, social phobia, posttraumatic stress disorder (PTSD), premenstrual dysphoric syndrome (PMDS), dysthymia, and seasonal affective disorder. There are six SSRIs that have been approved by the FDA in the United States, including citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline. All except fluvoxamine are approved by the FDA for use in depression (Sampson, 2001) even though fluvoxamine has been used as an antidepressant for many years in Europe (Goldberg, 2000).

All of the SSRIs have the same mechanism of action; they selectively inhibit the serotonin transporter (SERT) (Figure 2-3) (Goodwin, 1996). As is the case with almost all other antidepressants, the full onset of antidepressant action only starts after a few weeks. After acute administration of an SSRI, it inhibits SERT in the presynaptic serotonin neuron resulting in an increased concentration of serotonin in the synaptic cleft (Blier et al., 1987). It is only after chronic administration of an SSRI for two weeks or more that lasting high concentrations of 5-HT are achieved, explaining the slow onset of action. These high levels of 5-HT cause a desensitization of 5-HT<sub>1A</sub> autoreceptors, thereby reducing the 5-HT<sub>1A</sub> autoreceptor mediated inhibition of 5-HT release. This in turn increases the levels of 5-HT in the synapse and cause desensitization of the postsynaptic 5-HT receptors (Figure 2-3) (Blier et al., 1987; Blier et al., 1990; Chaput et al., 1991).

The SSRIs are associated with less adverse effects with comparable clinical efficacy when compared to other antidepressants (Ciraulo et al., 2011), rendering it the drug class of choice for current antidepressant therapy. The SSRIs are also the only FDA approved treatment for depression in pregnant women (fluoxetine) (Mulder et al., 2011:1961) and children and adolescents (fluoxetine and escitalopram) suffering from MDD (Bylund & Reed, 2007; Soutullo & Figueroa-Quintana, 2013; Wagner, 2005).

#### **2.1.7.4 The serotonin-norepinephrine reuptake inhibitors (SNRI)**

The SNRI's were specifically developed to be reuptake inhibitors of both 5-HT and NE, but without the side effects associated with the multi-potent TCAs. Drugs belonging to the SNRI class include venlafaxine, duloxetine and

milnacipran. Venlafaxine was the first to be introduced in this class and has been estimated to be more effective than the SSRIs (Bauer et al., 2009). Venlafaxine is the AD drug used in the current study and will be discussed in more detail.

The monoamine hypothesis remains the most extensively studied hypothesis of depression with robust construct validity, especially considering it has spawned the entire range of clinically effective ADs available today. According to this hypothesis both 5-HT and I-NE are strongly associated with MDD (Bylund & Reed, 2007; Montgomery, 1997). The SNRIs were specifically designed to target both these neurotransmitter pathways. However, as with the older drugs, such as the TCAs, have been proven to block both 5-HT and NE reuptake only at very high doses (de Oliveira et al., 2004; Entsuah et al., 2001; Gur et al., 1999), SNRIs also only become full dual reuptake inhibitors at higher dosages.

Venlafaxine is also a moderate dopamine (DA) reuptake inhibitor (Ellingrod & Perry, 1994), but at significantly lower levels than for 5-HT reuptake (Bolden-Watson & Richelson, 1993; Muth et al., 1986). Venlafaxine is also more selective for 5-HT reuptake inhibition than for the inhibition of I-NE reuptake (Bolden-Watson & Richelson, 1993; Béique et al., 1998; Muth et al., 1986; Owens et al., 1997) and is also a less potent inhibitor of the reuptake of I-NE than the TCAs.

It is also important to note that venlafaxine does not exhibit affinity for muscarinic-, cholinergic-, H<sub>1</sub>-, D<sub>2</sub>-, or benzodiazepine receptors (Cusack et al., 1994; Muth et al., 1986; Owens et al., 1997), thus giving it a better adverse effect profile than the MAOIs and the TCAs (Roseboom & Kalin, 2000).

It has been suggested in previous studies that the onset of action of venlafaxine is faster when compared to other classes of antidepressants (Feighner, 1994; Montgomery, 1997). This view is strengthened by other studies that have found venlafaxine to have a clinical response after only fourteen days, compared to the twenty one days for SSRIs (Benkert et al., 1996; Benkert et al., 1997; Clerc et al., 1994; Guelfi et al., 1995; Rudolph et

al., 1991). These faster actions have been attributed to its dual action on 5-HT and NA (Smith et al., 2002), although its onset of action is also under regulation by the restraining effect of the 5-HT<sub>1A</sub> receptor described earlier (Figure 2-3).

Venlafaxine also has analgesic effects and has been used in some cases to treat neuropathic pain (Enggaard et al., 2001), a response also mediated by its actions on I-NE and 5-HT. These monoamines are part of the centres in the brain that modulate the activity of the nociceptive pathway in the spinal cord (Sindrup & Jensen, 1999). Duloxetine has also been used to treat urinary incontinence (O'Donnell & Shelton, 2011).

### **2.1.7.5 The atypical antidepressants**

Drugs with unrelated chemical structures and mechanisms of action separate to those discussed above are referred to as atypical antidepressants. The atypical antidepressants were developed because of the constant search for novel ways to treat depression with drugs that have superior efficacy, better in tolerability and having a faster onset of action (Kent, 2000). The atypical antidepressants include the 5-HT<sub>2</sub> receptor antagonists (trazodone, nefazodone), alpha<sub>2</sub> receptor antagonists (mirtazapine), melatonergic receptor agonist (agomelatine), NE-reuptake inhibitors (reboxetine and maprotiline), 5-HT reuptake enhancers (tianepetine) and bupropion.

Trazodone and nefazodone (also mirtazapine) are all 5-HT<sub>2</sub> receptor antagonists, but also affect adrenergic and histamine H<sub>1</sub>-receptors as well (Eison et al., 1990; O'Donnell & Shelton, 2011). Mirtazepine inhibits the presynaptic α<sub>2</sub> receptors, impeding the body's natural control mechanisms for the release of neurotransmitters in the synapse (Figure 2-3) and is an antagonist of 5-HT<sub>2</sub>. Agomelatine is an agonist on melatonergic M<sub>1</sub> and M<sub>2</sub> receptors and a 5-HT<sub>2C</sub> receptor antagonist (San & Arranz, 2008). Reboxetine and maprotaline, which are both NE-reuptake inhibitors, exhibit antidepressant effects by increasing the levels of I-NE via inhibition of the norepinephrine reuptake transporter in the synaptic cleft (Figure 2-3) (Katona et al., 1999; Wong et al., 2000).

The tetracyclic antidepressant, tianeptine, has a unique and paradoxical mechanism of action, namely increasing the reuptake of 5-HT (Fattaccini et al., 1990; Ortiz et al., 1993), thus opposite in action as the SSRIs. It has also been found in recent studies that tianeptine buffers the excitatory amino acid receptors against stress (Kole et al., 2002) and this buffering protects the brain morphology from adverse effects associated with stress (McEwen & Magarinos, 2001). Tianeptine also affects the reuptake of I-NE and modulates MAO activity (Figure 2-3) (Brink et al., 2006). It has a better adverse effect profile than the above agents although has the same timeframe to the onset of action as the more conventional antidepressants (Wagstaff et al., 2001).

Bupropion acts to increase both noradrenergic and dopaminergic neurotransmission via inhibition of presynaptic NE and DA reuptake systems (Figure 2-3) (Foley et al., 2006), thus similar in action to TCAs, SSRIs and SNRIs.

Agomelatine is the most important new advance in antidepressant design and development. It is the only non-monoaminergic antidepressant currently clinically available and does not adhere directly to the monoamine hypothesis of depression. Even though the role of the different receptor activities of agomelatine in treating depression is still unknown, it is suggested that the antidepressant action is caused by synergism on both the melatonergic and 5-HT<sub>2C</sub> receptors (Papp et al., 2003).

#### **2.1.7.6 Delayed onset of action of antidepressants**

Patients that receive an antidepressant should be informed that there is a delay before the onset of action according to the National Institute for Health and Clinical Excellence (Tylee & Walters, 2007). This delay in the onset of action is important for several clinical reasons, because the delayed onset means that the disability associated with depression as well as the increased suicide risk continue until the full onset of action (Tylee & Walters, 2007). It is also important for patient compliance that early effects are visible. Unfortunately there is not a unifying definition that is agreed upon for onset of action and this impedes research in this field (Leon, 2000). In clinical practice there is particular truth in this, as it is often difficult to distinguish between

signs of response and side effects. It has been suggested by animal models that the acute biochemical changes stimulated by antidepressant treatment and the subsequent therapeutic action were due to the development of subsensitivity in the postsynaptic monoamine receptor (Vetulani & Sulser, 1975). These changes only became apparent after treatment with antidepressants over a similar period to that taken for clinical efficacy to develop.

## **2.2 Treating major depressive disorder in children and adolescents**

In the last two decades there has been a rapid growth of knowledge in the field of clinical pharmacology in general. On the contrary, there has been a scarcity of systematic research on treatment with antidepressants in children and adolescents with depression (Vitiello & Swedo, 2004). Children and adolescents have different developmental, biochemical, neurobiological, pharmacokinetic and pharmacodynamic characteristics when compared to adults and therefore the findings of research in adults cannot be applied directly (Vela et al., 2011). There is an urgent need to understand the different neural mechanisms between children, adolescents and adults, particularly as this could lead to the discovery of novel targets and more effective treatments (Mitchell et al., 2013).

Tricyclic antidepressants were found to be ineffective for the treatment of MDD in children and adolescents in a double blind, placebo-controlled study (Hazell et al., 2002). There were also reports of sudden deaths occurring as well as evidence of cardio toxicity in children and adolescents taking TCAs, and this discouraged clinicians from prescribing these medications to these age groups. Suitability of treatment in children and adolescents with MAOIs are limited due to the dietary restrictions and thus no data from clinical trials available (Ryan et al., 1988).

In the USA, up until recently, fluoxetine was the only drug approved by the FDA for treatment of MDD in children and adolescents aged 7-17 years (Bylund & Reed, 2007; Wagner, 2005). Escitalopram was however approved recently for treatment but only for adolescents aged 12-17 years (Soutullo &

Figuroa-Quintana, 2013). The current recommendation is to use a SSRI as mono therapy for first stage treatment, and if unsuccessful to switch to a different SSRI (Hughes et al., 1999). Earlier work on humans and animals concur that younger animals and children respond better to SSRIs than TCAs (Bylund & Reed, 2007). There is also a sound scientific rationale for this observation and for the specific approval and use of SSRIs in children, as will be discussed presently.

In younger patients the serotonergic system matures earlier than the noradrenergic systems, similar to that found in several mammalian species (Murrin et al., 2007). Hence, the clinical efficiency of the SSRIs is hypothesized to be the result of this faster maturation of the serotonergic system. The data on the safety and efficacy of antidepressants targeting the noradrenergic system, such as venlafaxine, in children are very limited (Andersen & Navalta, 2004) and studies of venlafaxine (fast and slow release formulations) have failed to find efficacy for these agents and have raised additional questions about their safety. A small published study of venlafaxine vs. placebo in 33 children and adolescents (8–17 years) treated with a relatively low dose (75 mg) found no difference between treatment and placebo (Mandoki et al., 1997). An 8-week study of venlafaxine XR vs. placebo in 334 children and adolescents with MDD (ages 7–17) also failed to find major differences between drug and placebo (Emslie et al., 2007). The reason for this lack of efficacy is interesting but can be directly attributable to age-dependent neurodevelopment.

Treatment duration of MDD in children and adolescents is similar to the adult condition and also requires a recommended twelve month period of treatment (Pine, 2002). Studies indicate an 82% response rate for children treated for twelve weeks, compared to a 75% response rate following six weeks of treatment (Emslie et al., 2004).

The current approach to the treatment of depression in children and adolescents can be summarised as follow: *'It is in the face of incomplete medical knowledge, with a wide range of scientific unknowns about safety and*

*the long-term developmental effects of drugs in the body, that we must practice paediatric psychopharmacology' (Vela et al., 2011).*

### **2.3 Results and findings in other studies relevant to the current project**

There are a number of pre-clinical and clinical studies available that are relevant to the specific objectives of the current study. Very few, however, involved an SNRI, such as venlafaxine, and most of the relevant studies focused on the effects of SSRIs in neuronal development and postnatal behaviour.

#### **2.3.1 Pre-clinical studies**

In a recent study it was found that daily injections of fluoxetine to pregnant mice increased the anxiety-like and depressive-like behaviour of the new-born pups when assessed in adulthood (Noorlander et al., 2008). An increase in anxiety-like and depressive-like behaviour was also reported after administering fluoxetine or citalopram to pups from ND+04 to ND+21, when tested later in life (Ansorge et al., 2008; Popa et al., 2008). Studies have also found that administration of citalopram to male rat pups from ND+08 to ND+21 reduced sexual activity (Maciag et al., 2005), decreased the latency to sleep onset and increased rapid eye movement (REM) sleep (Popa et al., 2008) later in adulthood. Paroxetine treatment from ND+33 to ND+62 has been shown to slightly elevate body weight, reduce sexual behaviour and to increase anxiety-like behaviour in animals when tested at ND+82 (de Jong et al., 2006). Another recent study showed that the atypical antipsychotic, risperidone, increased the 5-HT<sub>1A</sub>-receptor number in the medial prefrontal cortex and hippocampus of juvenile animals. In addition, there was a reduction in 5-HT<sub>2A</sub>-receptor subtypes at a relatively low dose of risperidone (Choi et al., 2010). From the preclinical data available one may postulate that chronic treatment with a centrally acting psychotropic during early-life development may affect normal neurodevelopment. These changes in neuronal development may cause effects that will only be visible later in life.

### **2.3.2 Clinical studies**

Fluoxetine is a widely accepted drug of choice for the treatment of MDD during pregnancy (Nonacs & Cohen, 2003). As mentioned before, fluoxetine and escitalopram (Soutullo & Figueroa-Quintana, 2013) are the only two drugs approved for the treatment of MDD in children and adolescents (Bylund & Reed, 2007; Wagner, 2005). Not surprisingly, therefore, clinical studies relevant to the current project have mainly reported on the use and effects of SSRIs in early-life.

The development of foetuses exposed to an SSRI was found to be different to that of foetuses not exposed, regardless of the SSRI type used. These differences included increased motor activity of the foetuses, measuring generalised foetal movement with real-time ultrasound observations, between weeks sixteen and nineteen of the first trimester as well as increased time spent in the activity phase between weeks twenty seven and twenty nine of the second trimester (Mulder et al., 2011). It has been proposed that this may be explained by a serotonergic neuron-mediated increase in motor neuron output that eventually facilitates repetitive movements of the foetus (Branchereau et al., 2002; Lucki, 1998).

No major effects on cognition, temperament or behaviour have been reported in infants who were exposed to SSRIs prenatally (Misri et al., 2006; Nulman et al., 2002; Oberlander et al., 2007).

## **2.4 Theoretical framework for enduring effects of drug action**

The main objective of the current project has been explained in Chapter 1. The current study set out to identify whether early-life administration of the antidepressant venlafaxine to rodents induces any long-term effects on behaviour or cognition, as well as on neurobiological markers of neurotransmission (5-HT and I-NE). In addition, the study aimed to determine at which later age the effects of early-life administration are most likely to be seen. There are several neurodevelopmental hypotheses that have been proposed to explain and predict such enduring effects of early-life antidepressant exposure.

The first hypothesis states that the process of overproduction and pruning of synapses in the developing brain could affect behaviour and neurobiological markers in later-life, as mentioned in §2.1.3. There are several studies available suggesting that mammals generally present with an overproduction of monoaminergic neurons, synapses and receptors during the developmental stage in life and that these levels decline to normal levels in adulthood (Andersen et al., 1997; Andersen et al., 2000; Whitaker-Azmitia, 1991). This process of decline is referred to as pruning, which can reach levels as high as 40% in the number of synapses lost during adolescence (Andersen et al., 2000; Peter, 1979). The processes of overproduction and pruning may cause vulnerability to long-lasting effects that are caused by psychotropic drugs that affect critically involved neuronal pathways in different regions of the brain (Andersen, 2003; Lidow & Song, 2001).

There are also two other hypotheses that have been suggested. The “neural Darwinism” hypothesis was introduced in the early 1990’s (Edelman, 1993). This hypothesis suggests that the brain “selects” the synapses that are to be retained into adulthood and this will allow the brain to match the needs of the environment (Piattelli-Palmarini, 1989; Teicher, 2002). The “instructionist” hypothesis, on the other hand, proposes that the environment “instructs” the brain to develop in a certain manner, based on the functional and/or structural requirements of the different brain systems (Quartz & Sejnowski, 1997). A number of studies have shown that synaptic development of the brain is influenced by changing levels of DA (Gelbard et al., 1990; Kalsbeek et al., 1988; Lankford et al., 1988; Todd, 1992), NE (Feeney & Westerberg, 1990; Kline et al., 1994) and 5-HT (Kuppermann & Kasamatsu, 1984; Lauder & Krebs, 1978; Whitaker-Azmitia & Azmitia, 1986) that occur during early-life. Being exposed to drugs that powerfully affect these neurotransmitters at a young age may cause lasting changes in the development of the brain that in turn will be manifest in various ways later in life (Andersen & Navalta, 2004).

## **2.5 Synopsis**

It can be seen from the literature described above that depression is a very serious and debilitating disease that does not discriminate with respect to age,

social status or race. It is also known to affect more and more people each year, while the rate at which prescriptions for these medications are increasing for children and adolescents is a matter of great concern. There is a lack of data on the safety and efficacy of currently available drugs, rendering minimal treatment options for clinicians to choose from. The effects of these drugs on the development of neurological pathways are mostly unknown while their associated effects on neurodevelopment following early life exposure urgently needs to be investigated. In fact, available data does suggest that psychotropic drugs administered early in life could affect neurodevelopment, including negatively, and that many of the long-term effects will manifest only later in life. This study aims to provide insight on the effects of early-life treatment with venlafaxine.

# Chapter 3 Materials and Methods

## 3 Chapter 3 Materials and methods

The different materials and methods used in the current study will be discussed in Chapter 3. This chapter will provide insight on the animals used, the methods of the behavioural tests as well as the methods for the determination of the *I*-NE and 5-HT concentrations and the statistical analysis employed. Before these tests were done, rats were treated as explained in the study layout and below.

### 3.1 Animals

The current study was ethically approved by the North-West University Ethics Committee (*approval number*: NWU-00045-10S5).

#### 3.1.1 The Flinders sensitive line rat as an animal model of depression

In the current study we used Flinders sensitive (FSL) and Flinders resistant line (FRL) rats. The FSL rat line was initially bred from Sprague Dawley rats by the Overstreet laboratory to display hyper-cholinergic activity (Overstreet et al., 1984), to provide resistance to the toxic effects of the organophosphates, in particular the irreversible cholinesterase inhibitor diisopropyl fluorophosphate (DFP) (Overstreet et al., 1979; Russell et al., 1982). The Overstreet team co-incidentally observed that these rats display depressive-like behaviour and this was followed up by validation of the FSL rat line as a genetic (inbred) animal model of depression (Janowsky et al., 1980; Overstreet et al., 2005). The FRL rats do not display hyper-cholinergic activity (resistance to DFP) as do FSL rats, and also do not display depressive-like behaviour relative to Sprague Dawley rats. Importantly, depressive-like behaviour of FSL rats is relative to that observed in the FRL (or Sprague Dawley) rats (Overstreet et al., 1979; Russell et al., 1982).

Validation criteria of animal behavioural models of human disease are well defined. It has to comply with criteria defined more than forty years ago by

McKinney and Bunney (McKinney Jr & Bunney Jr, 1969). The animal model must:

- be reasonably analogous to the human disorder in its symptomatology (face validity);
- cause behavioural changes that can be monitored objectively;
- produce behavioural changes that are reversed by the same treatment modalities that are effective in humans (predictive validity) and
- be reproducible between investigators.

The criteria for the use of an animal model of depression were simplified in 1995 by Geyer and Markou (Geyer & Markou, 1995) who stated that:

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

These authors also suggested that other criteria such as construct validity may be valuable, but is not essential for the model to have value as a potential tool for novel drug discovery and neurobiological research. Construct validity is the degree to which the animal model mimics the underlying neurobiology of the disease. Thanks to these simplified criteria several animal models have been developed since.

It is postulated that the depressive-like behaviour in the FSL rats is affected by the hyper-cholinergic characteristics of these rats, in line with the hyper-cholinergic super-sensitivity model of depression (Janowsky et al., 1980) described earlier in §2.1.4.2. This hypothesis can be seen as the first evidence of the construct validity of the model. In later studies it was suggested that there are additional correlates between the brain of depressed patients and the FSL rat line, such as modulated NO/cGMP signalling following stress (Harvey et al., 2006:201), serotonergic dysfunction (Overstreet et al., 1994;

Wallis et al., 1988; Zangen et al., 1999) as well as noradrenergic and dopaminergic dysfunction, compared to the FRL strain (Zangen et al., 1999). It is also important to note that chronic, but not acute, antidepressant treatment normalise the altered immobility in the FSL rats. (Dremencov et al., 2004; Overstreet et al., 1995; Overstreet, 1993; Zangen et al., 1997), adding to the predictive validity of the model.

The FSL rat line also presents with retarded psychomotor activity (Overstreet & Russell, 1982; Overstreet, 1986), reduced appetite and weight gain (Overstreet, 1993), alternated sleep patterns and immune abnormalities (Overstreet et al., 2005). It also demonstrates increased anxiety when performing certain tasks such as the social interaction test (Overstreet, 2002), but not in the elevated plus maze (Overstreet et al., 2005). The latter results suggest that enhanced anxiety may not be a prominent feature of the FSL strain (Neumann et al., 2011; Overstreet et al., 2005). All of these observations provide face validity of the model and correlates with the characteristics of depression in humans.

When comparing the FSL animal model of depression with the validation criteria of McKinney and Bunney mentioned above (McKinney Jr & Bunney Jr, 1969), the FSL rat line can be considered a robust, validated animal model of depression, with face-, predictive- and construct validity. It is also confirmed by different laboratories that have tested the model with objectively measurable parameters.

### **3.1.2 Limiting the study to male rats only**

The behaviour of female rats is affected by their hormonal cycles, which may affect behaviour and neurohormonal responses to pharmacological interventions. Previous studies found that female rats become sexually mature at about five weeks of age (ND+35) (Murrin et al., 2007; Zeinoaldini, 2005) and vaginal opening takes place around ND+32 and ND+34. The first sexual cycle occurs between thirty five and thirty seven days after birth (Moguilevsky et al., 1995), from which time hormonal cycles will commence. In the current study, animal behaviour was measured on postnatal day 35

(ND+35), 60 and 90, suggesting that female rats should be excluded. Therefore, the current study employed only male rats for behavioural and neurobiological testing.

### 3.2 Drug

The norepinephrine, serotonin reuptake inhibitor venlafaxine hydrochloride was used in the current study and was sponsored by Cipla Medpro, Cape Town, South Africa. It is described chemically as:

(RS)-1-[2-(dimethylamino)-1-(4 methoxyphenyl)-ethyl]cyclohexanol hydrochloride

or

(±)-1-[a [α- (dimethylamino)methyl] p-methoxybenzyl] cyclohexanol hydrochloride, empirical formula of C<sub>17</sub>H<sub>27</sub>NO<sub>2</sub> · HCl.

#### 3.2.1 Administration and dosage

Unborn foetuses received venlafaxine via administration to the pregnant mothers, whereas new-born pups were administered directly. When using chronic intraperitoneal (i.p.) administration to either the pregnant dam or the pup, they are vulnerable to injection injury. Alternative routes had to be explored. In a previous study it was found that i.p. administration only increased the bio-availability by 2-3% when compared to s.c. administration (Wright & Wilson, 1983). The conclusion can therefore be made that i.p. and s.c. administration routes may be considered to yield comparable drug levels. The benefit of s.c. administration is that it has reduced risk of injection injury. In light of the similarity in bioavailability, it is possible to determine a suitable dose for s.c. administration by using studies that implement i.p. administration. In this study all treatment regimens involved prenatal s.c. administration to pregnant dams as well as s.c. administration to new-born pups, both for two weeks (14 days). Animals received treatments prenatal:postnatal as follows: saline:saline (control), saline:venlafaxine, venlafaxine:saline or venlafaxine:venlafaxine. The pregnant dams were injected with 10 mg/kg s.c.

venlafaxine (Folkesson et al., 2010; Larsen et al., 2010; Scaini et al., 2010), whereas the pups received 3 mg/kg s.c. (Dawson et al., 1999).

In the current study male Flinders Sensitive Line (FSL) rats, and their corresponding behavioural control line, the Flinders Resistant Line (FRL) rats were used. During the prenatal phase of the study, pregnant female dams were used as carriers of the unborn male foetuses for the administration of drug or vehicle. For every treatment group one male rat was paired with one female rat of the corresponding genetic line for two nights. On the morning of the third day, the male rat was removed and this day was accepted as the day of conception, designated ND-21. The first series of daily treatment was started one week after the day of conception on ND-15 and ended fourteen days later on ND-01. A random selection of four new-born male pups were made from the litter of treated dams for the different groups and treatment of the pups was started on ND+03, ending fourteen days later on ND+17. The subjects were weaned on ND+21 and housed 4 rats/cage according to their groups under normal conditions (see below) until the day of behavioural testing i.e. ND+35, 60 or 90.

### **3.2.2 General housing protocol**

All animals were housed under conditions of constant temperature ( $22 \pm 1^\circ\text{C}$ ) and humidity (50%) with a 12:12-h light/dark cycle (lights on 06:00 to 18:00). Food and water were provided ad libitum.

The study and all animal procedures were in accordance with the guidelines given for the care and use of laboratory animal by the National Institutes of Health.

### **3.3 Behavioural tests**

The behavioural and memory tests described below were performed on ND+35, 60 or 90 for all groups of rats. All behavioural tests were performed sequentially between 1 to 4 hours after the start of the dark cycle (i.e. 19:00 – 22:00). Tests implemented include the novel object recognition test (nORT), locomotor assessment using the Digiscan® animal activity monitor (DAAM),

the elevated plus maze (EPM) and the forced swim test (FST), in this order. Animals were relocated to different rooms for the above testing. Between each test a period of 30 minutes was allocated for acclimatisation before being subjected to a specific test. The order of the tests was designed to start with the least stressful interventions and to end with the most stressful interventions. Importantly, a pilot study in our laboratory verified that preceding tests in the test battery do not significantly affect any of the subsequent tests (Mokoena, 2013, unpublished data). Typical data from this pilot study are presented in Table 3-1 below. From these data it can be seen that the order in which we did the battery of tests had no significant effect on the immobile behaviour shown by the rat in the FST.

**Table 3-1:** Immobility shown by rats in the FST after a battery of behavioural tests.

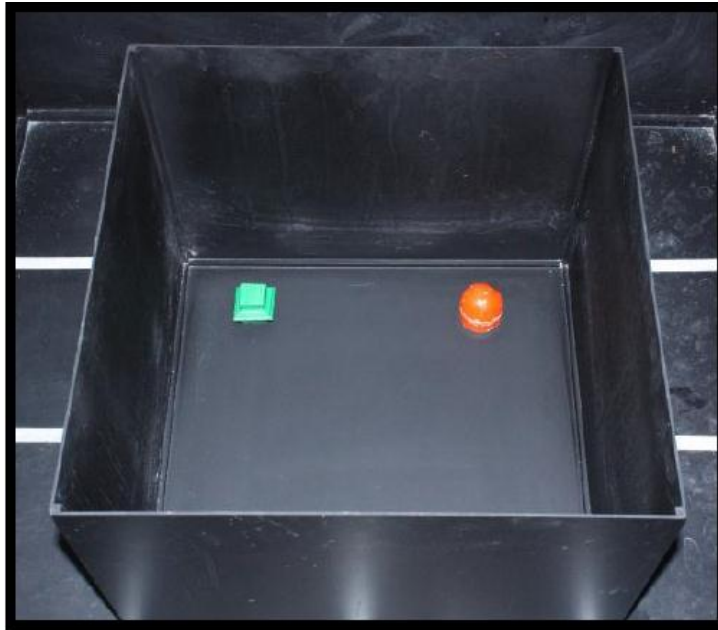
<b>Test battery</b>	<b>Test order</b>	<b>Immobility (seconds), as measured in the FST (Means <math>\pm</math> SEM)</b>
1	LCM + FST	154.4 $\pm$ 7.161
2	NORT + LCM + EPM+ FST	153.1 $\pm$ 7.376
3	NORT + SIT+ LCM + EPM+ FST	147.1 $\pm$ 9.503

Values are means  $\pm$  standard error of the mean (SEM) for 8 rats per group. LCM = locomotor, FST = forced swim test, nORT = novel object recognition test, EPM = elevated plus-maze, SIT = Social Interaction Test.

From these data it has thus been demonstrated that preceding tests do not affect the outcome of the forced swim test performed at the end.

### **3.3.1 The novel object recognition test**

The novel object recognition test (nORT) was first described over twenty years ago (Ennaceur & Delacour, 1988). Since then it has been used to examine animal memory performance. The novel object recognition test has since been used extensively although various modifications of the original test have been implemented. The test has one major advantage in that it requires no aversive or stressful stimuli to be applied for the test to be effective (Rutten et al., 2008), unlike the Morris water maze that involves swim stress.



**Figure 3-1:** The novel object recognition test box, as implemented in the current study. The photograph illustrates the four opaque walls as well as the two different immovable objects.

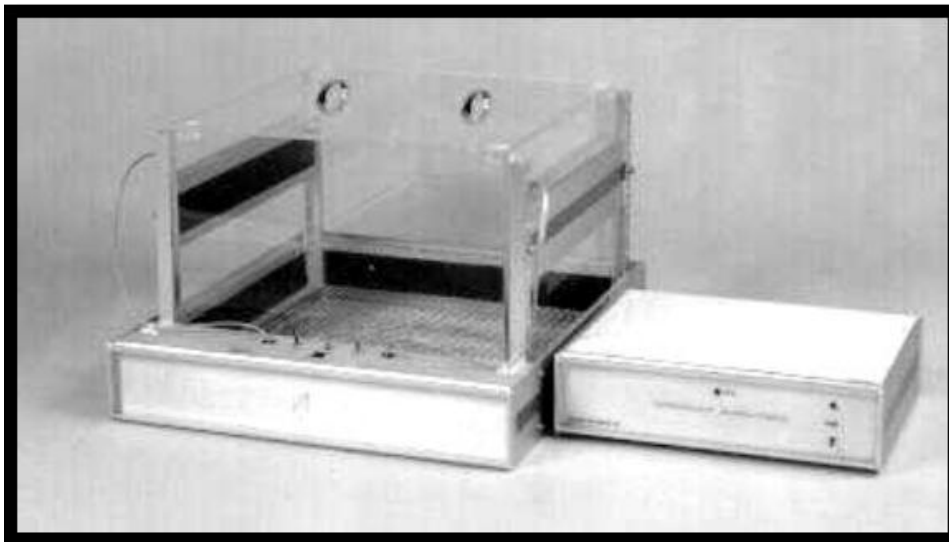
The natural tendency of the animal to explore novel objects relative to a familiar one is used to determine the memory performance in the nORT (Rutten et al., 2008). It is therefore expected that the rat with intact memory will spend more time exploring a novel than a familiar object. If a rat spends equal time exploring the novel object compared to the familiar one during the retention trial, impaired memory function is observed.

After a 30 minute acclimatization period, the test was performed under low light intensity (20 lx) and each rat was placed in a separate nORT box (dimensions L x W x H = 50 x 50 x 40 cm) (compare Figure 3-1). The rat was placed in the centre of the box facing the objects, with the objects spaced approximately 20 cm from the walls of the boxes, each placed in an adjacent corner (compare Figure 3-1). First an acquisition trial was performed where the rats were left to explore the nORT box with two identical objects (red balls) for five minutes. The rats were then returned to their cages for 90 minutes before the onset of the retention trial. The nORT boxes were cleaned with a 10% ethanol solution to eliminate any olfactory cues from the previous test. After 90 minutes the rats were returned to the nORT boxes for the retention

trial, where one of the familiar objects (red ball) was now exchanged for a novel object (green block). The rats were again left to explore the nORT boxes for 5 minutes, during which the time spent exploring the familiar and novel object was recorded on video for later scoring. Exploration of the objects was defined as licking, sniffing or physically touching the object.

### 3.3.2 The Digiscan® animal activity monitor

The Digiscan® animal activity monitor (DAAM) provides automated and continual computerized monitoring of animal movement. The sensitivity is much higher than visual observation and it removes the risk of investigator bias (Sanberg et al., 1983; Sanberg et al., 1987).



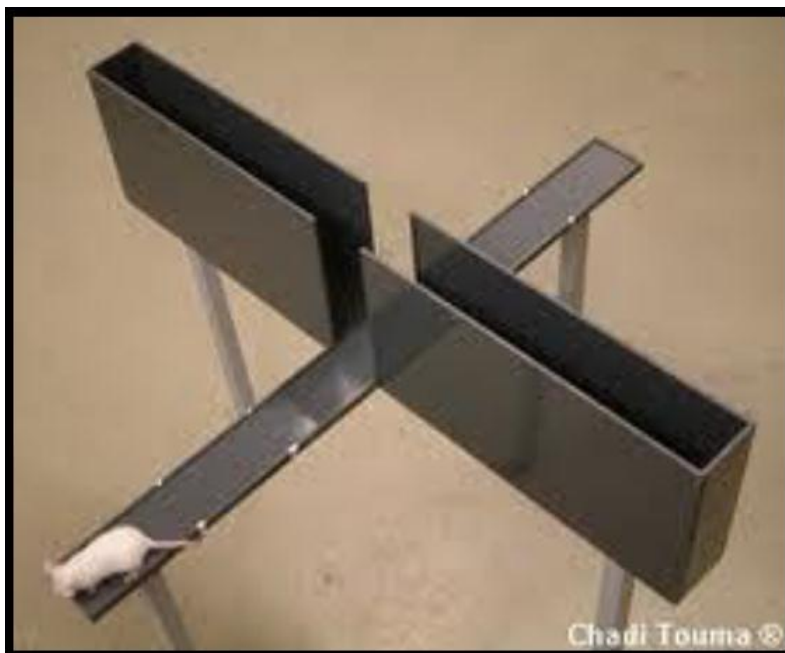
**Figure 3-2:** The Digiscan® animal activity monitor, as implemented in the current study.

The cages of the DAAM (Fig 3-2) have a series of cross-sectional horizontal infrared light beams at ground level as well as additional beams 10 cm above the first. The computerised collection of the locomotor activity of the rats is enabled by these infrared beams and accurately provides a profile of all aspects of the animal's vertical and horizontal movements. By breaking any one of the beams, the action is interpreted as an activity score and by breaking two or more consecutive beams, the action is recorded as a movement score (Korff et al., 2008). During the current study horizontal activity (number of beam breaks) were recorded during a five minute trial. The

rats were placed individually in the centre of separate DAAM cages and left to explore for five minutes to acquire the above mentioned parameters.

### 3.3.3 The elevated plus maze

The elevated plus maze (EPM) was developed to identify the extent of anxiolytic and anxiogenic effects of drugs. It has also been successfully used to study behavioural changes later in life after early-life manipulations (Estanislau et al., 2011; Hinojosa et al., 2006). It was first used to study the involvement of noradrenergic system in anxiety by Handley and Mithani in the late 1980's (Handley & Mithani, 1984). This well-described, standard test consists of a cross-shaped apparatus with two enclosed (i.e. walled) and two open (i.e. unwalled) arms (Figure 3-3). The apparatus consists of a plus-shaped maze (1 m x 1 m and 10 cm arm width), elevated 50 cm from the floor (Fig 3-3).



**Figure 3-3:** The elevated plus maze, as implemented in the current study. The photograph illustrates the plus-shaped platform, elevated from the floor surface as well as the two enclosed arms.

Rodents have a natural tendency to seek the shelter of an enclosed space (aversion of predation etc.), but their curiosity drives them to explore the open areas. The test is based on these natural tendencies and the balance of time

spent in exploration versus shelter is dependent on the animal's inherent anxiety state. The more anxious the animal is, the more it will seek the protected environment of the closed arms, and the less anxious, the more it will spend time exploring on the open arms. This can then be used to determine the anxiety of an animal as well as the extent of anxiogenic and anxiolytic effects of a psychotropic drug (Pellow et al., 1985).

After a 30 minute acclimatization period each rat was placed in the centre of the EPM within a visually enclosed area, facing an open arm. The rats were left to explore the EPM for five minutes and were recorded with a video camera mounted above the EPM. The videos were then scored and the time spent in the open and closed arms analysed. After each test olfactory cues were removed by wiping the EPM with a 10% ethanol solution. In the current study the standard EPM was used to measure anxiety-like behaviour under a light intensity of 80 lx measured in the open arm and 20 lx in the closed arm, as described previously (Carola et al., 2002:49). The percentage time spent in the open arms was calculated, as well as the number of entries and full entries into the open arms counted. A full entry was defined as crossing into the open arm section with all four paws.

#### **3.3.4 The forced swim test**

The forced swim test (FST) was originally developed in the 1970's to screen for antidepressant activity in rodents by assessing increases and decreases in immobility during a forced swim trial (Porsolt et al., 1977). The FST is based on the observation that when rats are placed in an inescapable cylinder of water that after initial escape-orientated movements, the rats will develop an immobile posture (conditioning trial). This immobile posture is resumed quickly if the test is performed again 24 hours later. This observation of immobility is believed to reflect a failure of persistence in escape-directed behaviour or the development of passive behaviour that helps the animal to cope with stressful stimuli by disengaging.

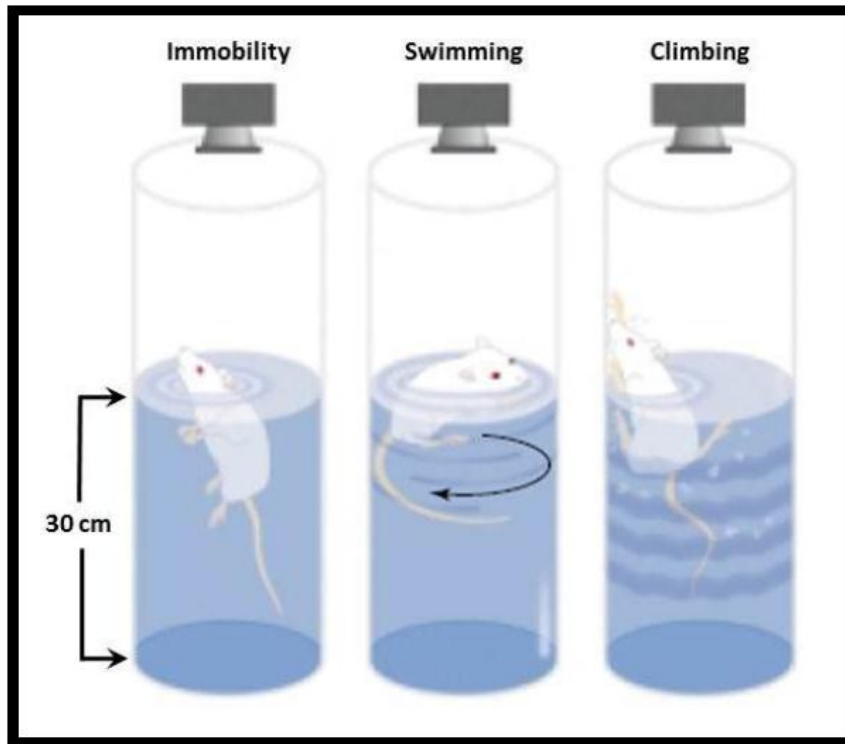
The test has undergone various modifications since its first introduction, including the water depth and scoring of additional parameters, now including

immobility, swimming and climbing. It was later modified for distinguishing between serotonergic and noradrenergic mechanisms (Lucki, 1997:523) involved in drug action. The FST is a very robust test and is a well validated method for measuring depressive-like behaviour following an intervention. It has predictive validity for detecting the activity of a broad range of antidepressant classes and it has become a standard screening tool for antidepressant drugs and/or behaviour (Liebenberg et al., 2010).

The FST is based on the observation that rats develop an immobile posture when exposed to an inescapable cylinder of water, following initial escape-directed movements that are extinguished during a re-swim 24 hours later. If an antidepressant drug is administered in an acute regimen to these animals immediately following the first conditioning trial, they persist in engaging escape-directed movements in the second trial on day two for longer periods of time, as compared to the vehicle-treated controls (Liebenberg et al., 2010). It is important to note that FSL rats display inherent depressive-like behaviour and that there is no need for a conditioning trial (Porsolt et al., 1977).

Four FST cylinders (60 cm high and 24 cm in diameter) were filled with 30 cm of water and maintained at 25°C before the start of the test. Each rat was placed in a separate cylinder and left to swim for a total of seven minutes. The time spent in the cylinder was recorded using a video camera mounted across the cylinders. After the seven minutes passed the rats were removed and dried with a paper towel and returned to their home cage. Each cylinder was washed with 10% ethanol solution and rinsed before the next group of rats were subjected to the FST.

By scoring immobility, climbing and swimming behaviour separately, it is possible to distinguish serotonergic and noradrenergic mediated mechanisms of antidepressant action. In this scenario, noradrenergic mechanisms will increase climbing, whereas serotonergic mechanisms will favour swimming behaviour (Cryan et al., 2002) (Figure 3-4). In the final results the first and last minute of the seven minutes spent swimming was omitted. This means that only five minutes were scored and this increases the accuracy of the results. The various behaviours scored in the FST are as follows:



**Figure 3-4:** Illustration of the swimming, climbing and immobility behaviour of the FRL and FSL rats during the forced swim test, as implemented in the current study (Cryan et al., 2002).

- Immobility is defined as in the traditional Porsolt test (Porsolt *et al.*, 1977), when no active movements are made, except that needed to keep the rat's head above the water;
- climbing (or struggling) behaviour is defined as upward-directed movements of the forepaws along the inside of the swim cylinder; and
- swimming behaviour is defined as horizontal movements throughout the cylinder that include crossing into another quadrant (Liebenberg *et al.*, 2010).

### 3.4 Neurochemical analysis

In the current study we determined the levels of serotonin (5-HT) and l-norepinephrine (l-NE) in crude homogenates of rat brain, but specifically focusing on the prefrontal cortex and hippocampus. The analysis was undertaken using High Pressure Liquid Chromatography coupled with

Electrochemical Detection (HPLC-ECD). The original method used was originally developed in our laboratory by Basson (Basson et al., 1988) and has since been modified with various improvements by Harvey et al (Harvey et al., 2006). Several changes were made in the mobile phase, the HPLC column and settings for the electrochemical detection, but the essence of the method remained the same.

### 3.4.1 Chromatographic conditions

**Table 3-2:** Chromatographic conditions of the method

<b>Analytical instrument:</b>	Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem III Electrochemical detector with a coulometric flow cell and Chromeleon® Chromatography Management System version 6.8.
<b>Column</b>	Synergi 4 $\mu$ Hydro-RP, 250 x 4.6 mm, 4 $\mu$ , 80 Å pores, Phenomenex, Torrance, CA .
<b>Guard column</b>	SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18, 4.0 x 3.0 mm, Phenomenex, Torrance, CA.
<b>Mobile phase</b>	0.1 M sodium formate buffer (6,801 g/l), 5 mM Sodium 1-heptanesulfonate (1,01125 g/l), 0.17 mM ethylenediaminetetra- acetic acid disodium salt (20 mg/l), v/v 5.5% acetonitrile and 1.5% methanol. The pH of the mobile phase was set at $\pm$ pH 3.85 with orthophosphoric acid (H <sub>3</sub> PO <sub>4</sub> ) (85%).
<b>Flow rate</b>	1.00 ml/min
<b>Injection volume for instrument</b>	20 $\mu$ l
<b>Coulometric electrochemical detector settings for instrument</b> E1 – Test Electrode 1 E2 – Test Electrode 2 EGC – Guard Cell	Settings for the coulometric flow cell: Model 5011A High Analytical Cell: Cell Potential Settings: E1 = -150 mV, E2 = +750mV, EGC = +150mV, Range: 20 nA, Filter: 0.5 sec, Offset: 0%, Signal output: 1.0 V.
<b>Relative retention times</b>	Norepinephrine (NE) $\pm$ 6.5 minutes.  Isoprenaline Internal Standard $\pm$ 27.9 minutes.  Serotonin (5-HT) $\pm$ 50.7 minutes

### 3.4.2 Reagents

#### Solution A

Contents:

0.5 mM sodium metabisulphite (E Merck, Midrand).

0.3 mM ethylenediaminetetra-acetic acid disodium salt (Na<sub>2</sub>EDTA) (E Merck, Midrand).

0.25 M perchloric acid (HClO<sub>4</sub>) 60% strong solution (E Merck, Midrand).

Preparation:

Weigh 0.047525 g sodium metabisulphite and 0.055836 g Na<sub>2</sub>EDTA and dissolve it in 400 ml distilled water.

Add 13.587 ml perchloric acid to above solution and make up to 500 ml with distilled water.

Note: All standards were prepared in this solution (solution A).

Preparation of standard solutions

#### 3.4.2.1 Norepinephrine (NE)

*l*-Norepinephrine hydrochloride = 205.6407 MW

Norepinephrine = 169.1798 MW (82.27%)

Weigh of 1.22 mg and dissolve it in solution A, 82.27% of the 1.22 mg will be 1 mg that will represent norepinephrine.

#### 3.4.2.2 Serotonin (5-HT)

Serotonin creatinine sulphate or 5-Hydroxytryptamine creatinine sulphate = 405.43 MW

Serotonin = 176.2 MW (43.46%)

Weigh 2.30 mg and dissolve in solution A, 43.46% of the 2.30 mg will be 1 mg and represents serotonin.

All the above analytes can be dissolved together in 10 ml of solution A in a 10 ml amber volumetric flask. This solution will be the standard stock solution and all the analytes will give a concentration of 100 µg/ml. From this solution a range of concentrations can be prepared to setup a standard curve.

All of the working standards will be prepared from this solution, see table below.

**Table 3-3:** Preparation of standard solutions

<b>Working Standards</b>	<b>Concentration (ng/ml)</b>	<b>Dilution</b>	<b>+</b>	<b>Solution A</b>	<b>=</b>	<b>Total Volume</b>
<b>1</b>	<b>1</b>	20 µl (B)	<b>+</b>	1980 µl	<b>=</b>	2 ml
<b>2</b>	<b>2.5</b>	50 µl (B)	<b>+</b>	1950 µl	<b>=</b>	2 ml
<b>3</b>	<b>5</b>	100 µl (B)	<b>+</b>	1900 µl	<b>=</b>	2 ml
<b>4</b>	<b>7.5</b>	150 µl (B)	<b>+</b>	1850 µl	<b>=</b>	2 ml
<b>5</b>	<b>10</b>	200 µl (B)	<b>+</b>	1800 µl	<b>=</b>	2 ml
<b>6</b>	<b>15</b>	300 µl (B)	<b>+</b>	1700 µl	<b>=</b>	2 ml
<b>7</b>	<b>20</b>	400 µl (B)	<b>+</b>	1600 µl	<b>=</b>	2 ml
<b>B</b>	<b>100 ng/ml</b>	200 µl (A)	<b>+</b>	9800 µl	<b>=</b>	10 ml
<b>A</b>	<b>5 µg/ml</b>	100 µl (SS)	<b>+</b>	1900 µl	<b>=</b>	2 ml

### 3.4.3 Preparation of internal standard (I.Std)

#### a) Isoprenaline

R/S-Isoproterenol hydrochloride

Weigh off 1 mg and dissolve it in 10 ml of solution A, this solution will be the stock solution for the internal standard. Take 30µl of the stock solution and make up till 2 ml with solution A, this solution will be the working internal standard solution.

This final concentration will be the working internal standard solution with a concentration of 1500 ng/ml.

#### 3.4.4 Sample preparation of brain tissue samples

1. Following appropriate dissection of the rat brain, the tissue of each animal was placed individually into Eppendorf® tubes, marked and frozen with liquid nitrogen and stored at -80°C.
2. On the day of analysis, the brain sample was weighed and 1 ml of solution A was added to the tube. The tissue in the tube was then ruptured by sonication (2 x 12 seconds, at an amplitude of 14  $\mu$ ), which is the preferred method of cell disruption for small sample and volume size.
3. The tube was allowed to stand on ice for 20 minutes to complete perchlorate precipitation of protein and extraction of monoamines.
4. Following this period, the sample was centrifuged at 4°C in an ultracentrifuge for 20 minutes at 16 000 rpm (24 000 g).
5. The supernatant fluid (tissue extract) was removed and pipetted into a 2 ml amber Eppendorf® tube.
6. The pH of the sample was adjusted to pH 5 with the addition of 1 drop/ml of 10 M potassium acetate.
7. An aliquot of 200  $\mu$ l of the tissue extract was pipetted into a 1.5 ml Eppendorf® tube.
8. Then 20  $\mu$ l of the internal standard, isoprenaline, was added to the sample.
9. The final sample was vortexed and centrifuged for 5 minutes at 14 000 rpm (21 000 g).
10. Then 200  $\mu$ l was pipetted into a 300  $\mu$ l glass insert that fits into a HPLC vial.
11. The instrument's software was programmed to inject 20  $\mu$ l onto the HPLC column, and analyse NE and 5-HT at E2 (Testing electrode 2) with setting at +750mV.

12. The results were expressed as ng/mg (nanograms per milligram) of wet weight tissue.

### **3.5 Statistical Analysis**

Analyses was undertaken with an unpaired Student's T-test for direct comparison between two groups, while multiple comparisons with one variable per analysis was analysed using the one-way ANOVA with a Tukey post-hoc test. Differences were assumed statistically significant when  $p < 0.05$  (\*). GraphPad Prism® (version 6.00, San Diego California, U.S.A.) was used for these statistical analyses. Data that did not show statistical significance with the above mentioned method of analysis were tested for practical significance using the Cohen's test. Practical significance was assumed when  $d \geq 0.5$  (#) and  $d \geq 0.8$  (##).

# Chapter 4 Results and Discussion

## 4 Results and Discussion

In this chapter the results obtained from the study will be discussed. The data in this section is a combination of data from the current and a previous study and was combined to try and increase the statistical power. The specific pre- and postnatal treatment regimen used in each group will be designated in the results as follows (“D”=“drug” and “C”+“control”):

Prenatal	Postnatal	Designation
vehicle control	vehicle control	CC
vehicle control	venlafaxine	CD
Venlafaxine	vehicle control	DC
Venlafaxine	venlafaxine	DD

There were unfortunately no pups born for the ND+90 CD group and accordingly no data is available on any of the tests for this group.

Statistical analyses were done using the unpaired Student’s T-test for direct comparison between two groups, or multiple comparisons with one variable per analysis using the one-way ANOVA followed by the Tukey posthoc test as explained in Chapter 3.

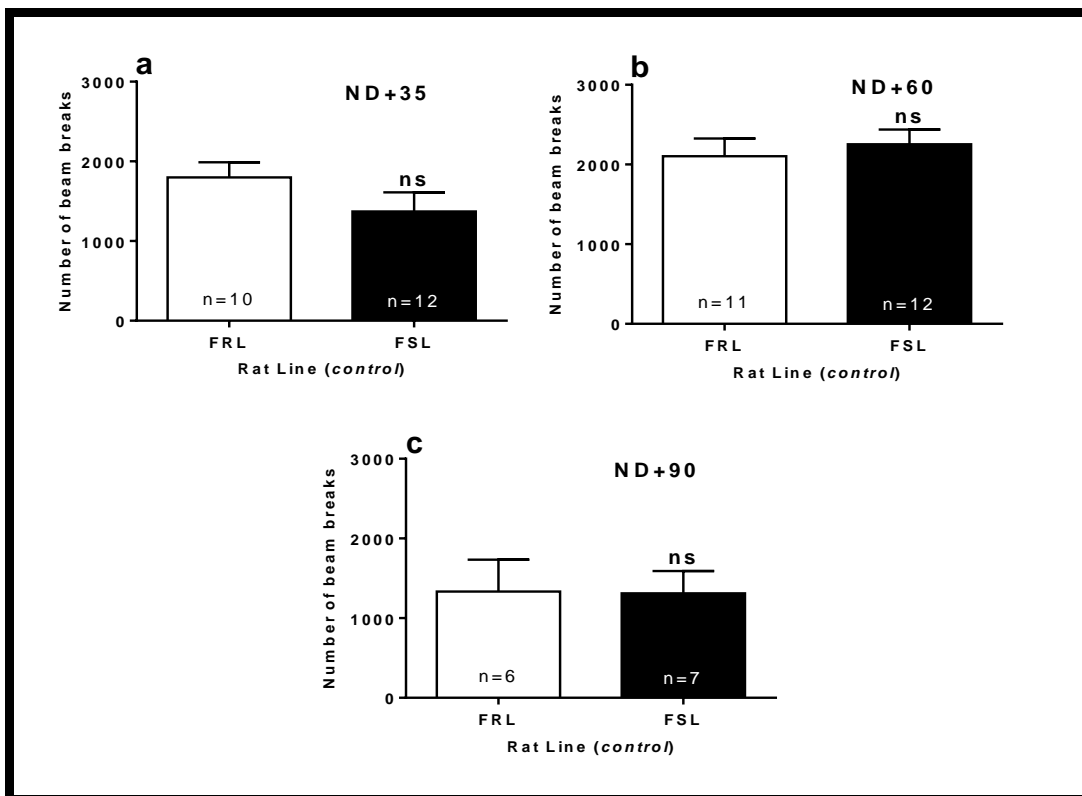
Importantly, the results from the current M.Sc. study were compromised by renovations at the animal centre at the North-West University. The rats were housed under suboptimal conditions due to stressors that were present during housing, consequently introducing the risk of adverse effects in the rats that could adversely affect the data. This undesirable yet unavoidable event resulted in poor breeding, leading to insufficient animal numbers in certain test groups, and hence poor statistical power of the data. The stress also negatively affected behaviour in the animals, so that the reliability of the data may have been compromised to some extent.

## 4.1 Locomotor activity

The locomotor activity of the rats was determined using the Digiscan® Animal Activity Monitor (DAAM) as explained in *Chapter 3*. The number of beam breaks (horizontal activity) made by the rats were determined in the test over a five minute trial.

### 4.1.1 FRL Control vs. FSL Control

Firstly it was important to understand whether drug-naïve FSL and FRL rats displayed similar or different locomotor activities. Therefore we determined the locomotor activity of the FRL CC and FSL CC groups, as depicted in Figure 4-1:



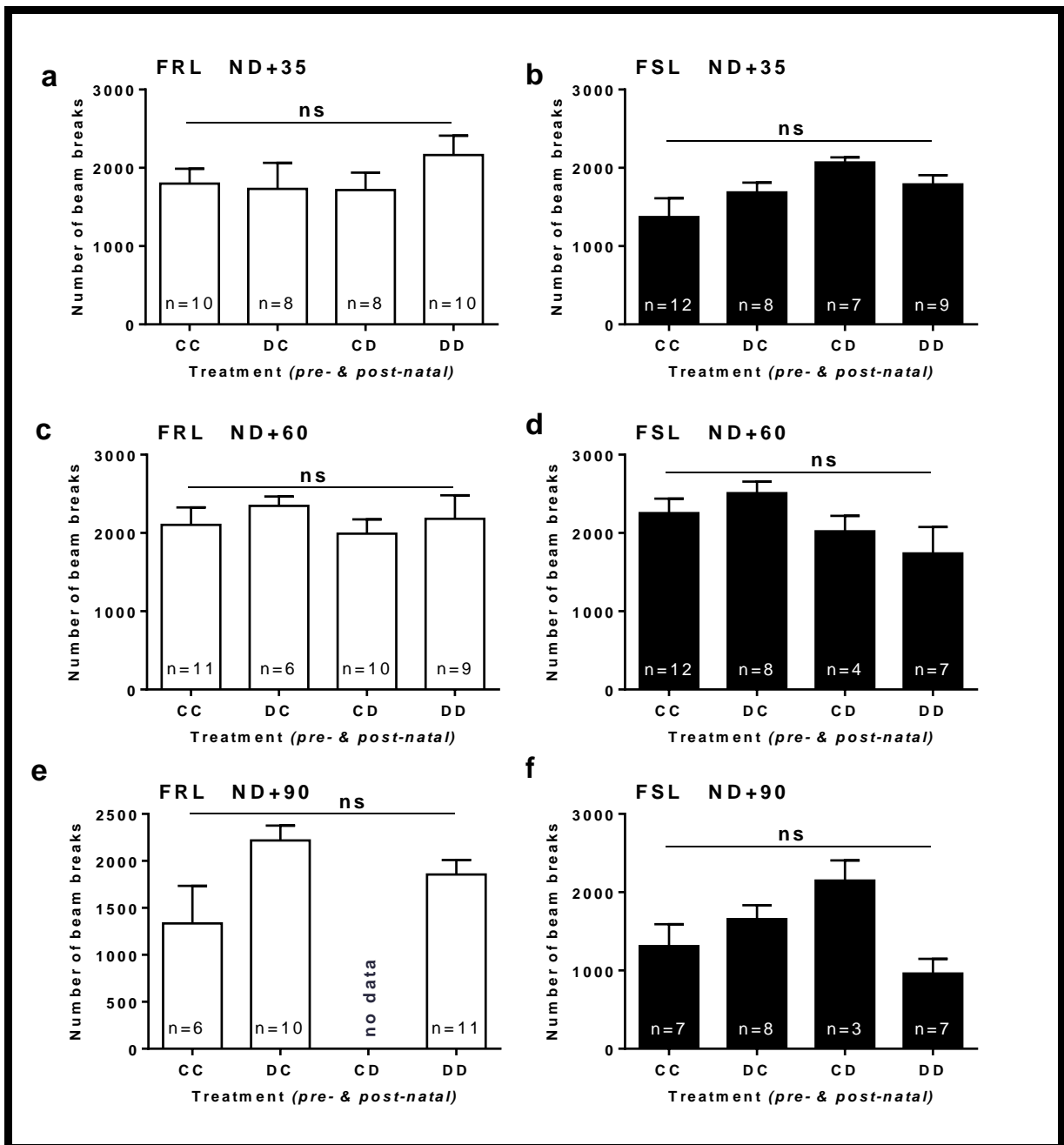
**Figure 4-1:** Number of beam breaks in the Digiscan® animal activity monitor by FRL and FSL control groups on the different specified ages postnatal. FRL and FSL control groups at postnatal day 35 (a), 60 (b) and 90 (c). The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ).

The data presented in Figure 4-1 shows that in the current study there were no significant changes in locomotor activity between vehicle-treated FRL and

FSL rats at ND+35, ND+60 or ND+90. Since there is no difference in the locomotor activity shown by the FRL and FSL rats at any of the specified ages, it suggests that differences in immobility data of FSL versus FRL rats in the FST cannot be attributed to altered locomotor activity (Dawson & Tricklebank, 1995).

#### **4.1.2 Effects of early-life venlafaxine administration**

The data presented in Figure 4-2 shows the effects of the different treatment regimens on the horizontal activity of the animals as determined in the Digiscan Animal Activity Monitor over a five minute trial.



**Figure 4-2:** Number of beam breaks in the Digiscan® animal activity monitor by FRL and FSL rats that received the specified venlafaxine treatment regimen in early-life phases at postnatal day 35 (a, and b), 60 (c, and d) and 90 (e, and f). The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ).

Statistical analyses of the data in Figure 4-2 did not reveal any statistically significant differences in locomotor activity between any of the treatment groups. Although differences cannot be excluded due to the statistical

weakness of the data, this was expected and most likely a true reflection of the pharmacological treatment effects. Therefore, any differences in immobility data between treatment groups in the FST cannot be attributed to altered locomotor activity (Dawson & Tricklebank, 1995).

## **4.2 Forced Swim Test**

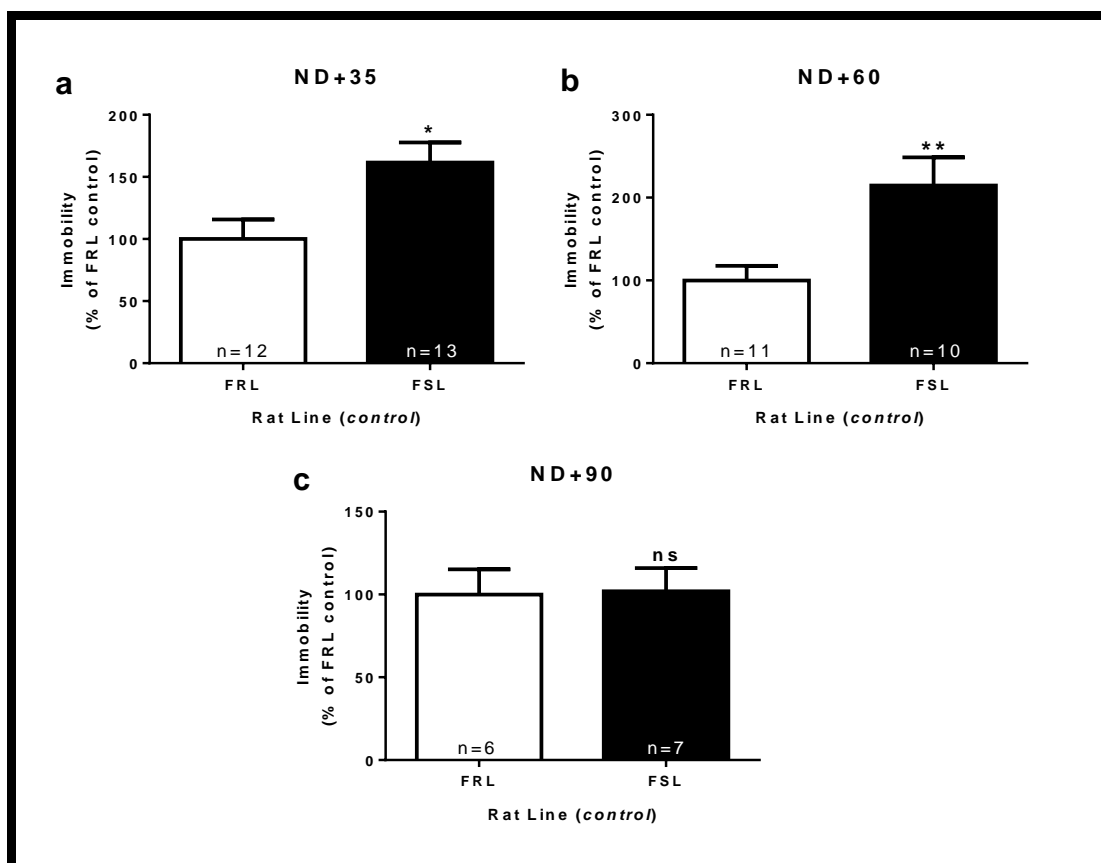
In the current study we used Flinders sensitive line (FSL) rats as a well-described and validated rodent model of depression (see Chapter 3) and their control rat line, the Flinders resistant line (FRL) rats. Since the data from the current study was added to data from a previous study that also evaluated these rats at ND+60, it was corrected for inter-experiment and inter-experimenter variation by converting the data to percentage of control.

### **4.2.1 Immobile behaviour**

Immobile behaviour shown by the rat in the FST is a measure of depressive-like behaviour. It has been observed in several studies that FSL rats are more immobile in the FST than their FRL counterparts (Overstreet, 1986; Overstreet et al., 1992), including earlier work performed in our own laboratory (Liebenberg et al, 2010).

#### **4.2.1.1 FRL control vs. FSL control**

It was important to understand whether drug-naïve FSL and FRL rats displayed similar or different depressive-like behaviour. Therefore we determined the depressive-like behaviour as a percentage of the time spent immobile in the FST by the FRL CC and FSL CC groups, as depicted in Figure 4-3.

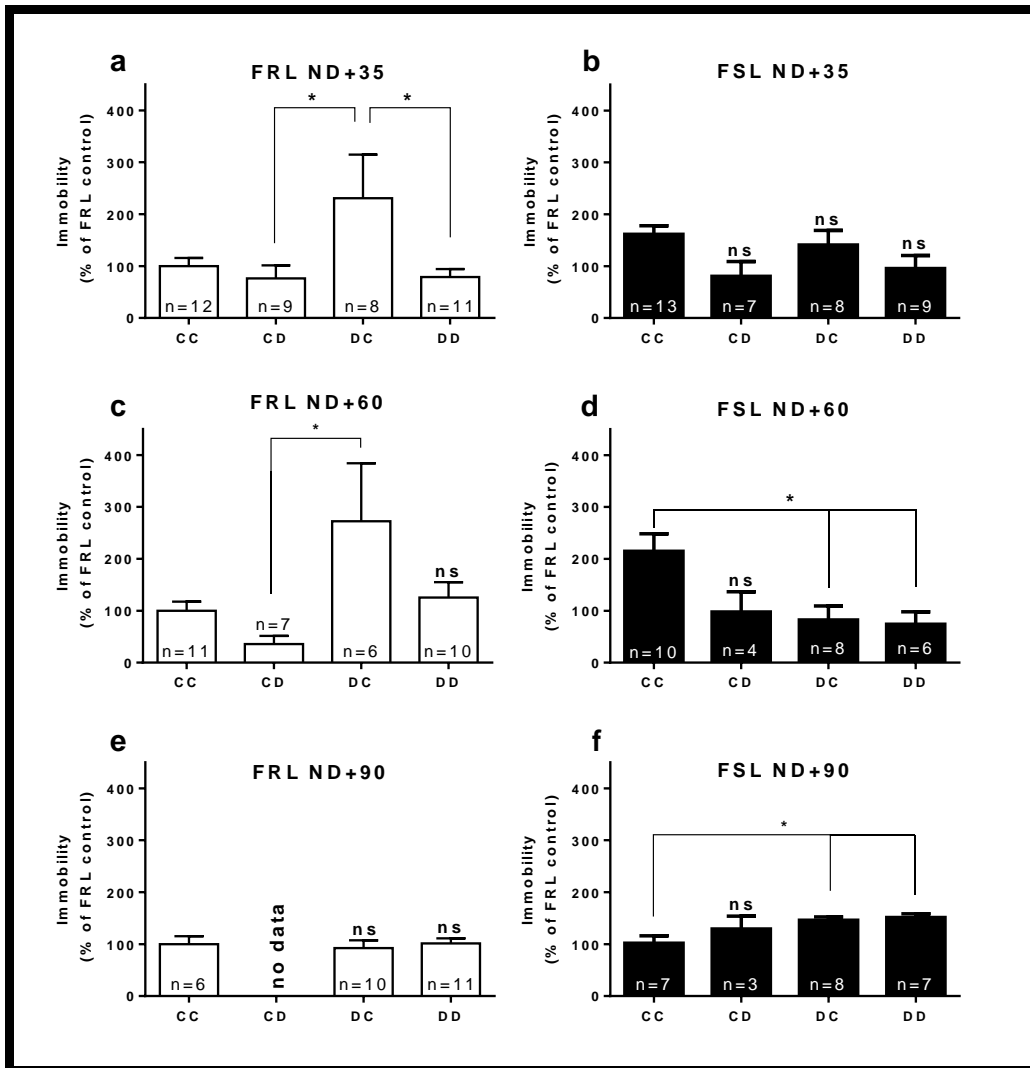


**Figure 4-3** Percentage time spent immobile in the FST by FRL and FSL control groups at postnatal day 35 (a), 60 (b) and 90 (c). The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ) and \*\* = significant (i.e.  $p < 0.01$ ).

Figure 4-3 shows that drug-naïve FSL rats displayed a statistically significant increase in immobile behaviour when compared to their FRL counterparts. These data confirm that the FSL rats indeed displayed significant depressive-like behaviour relative to FRL rats under current experimental conditions at ND+35 and ND+60. It has been observed in several studies that the FSL rats are more immobile in the FST than their FRL counterparts (Overstreet, 1986; Overstreet et al., 1992). It should be noted, however, that the vehicle-treated FSL rats at ND+90 did not display enhanced immobility relative to the corresponding FRL rats, suggesting that the depressive-like responses of the stress-sensitive rat line was lost by this age (§4.2.1.1).

#### 4.2.1.2 Immobile behaviour relative to age and following venlafaxine treatment

The effect of the pre- and/or postnatal treatment with venlafaxine on immobile behaviour of the rats, as seen at ND+35, ND+60 and ND+90 is depicted in Figure 4-4.



**Figure 4-4:** Immobile behaviour shown during the FST by FRL and FSL rats following venlafaxine treatment at postnatal day 35 (a, and b), 60 (c, and d) and 90 (e, and f). The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ).

The results in Figure 4-4 suggest that there is a significant increase in immobility during adolescence in the FRL rats at ND+35 following prenatal

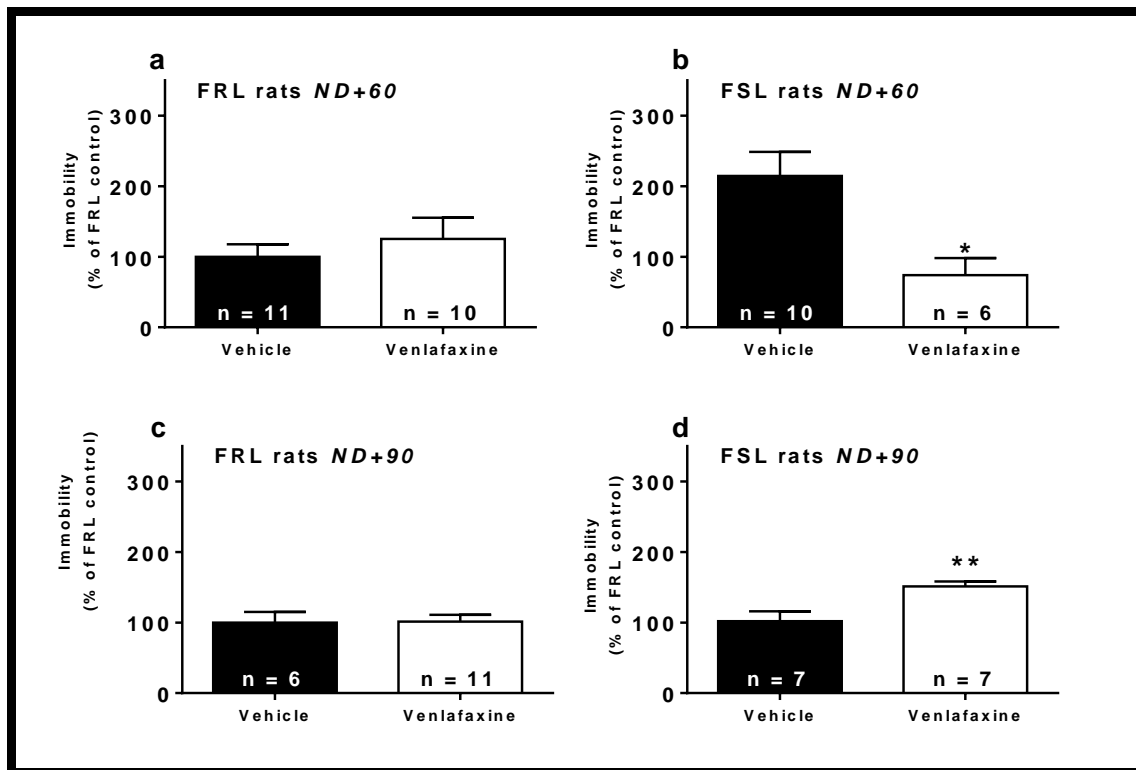
treatment with venlafaxine and postnatal treatment with the vehicle. This suggests an increase in depressive-like behaviour in this group, but this increase in immobility is then reversed if the treatment with venlafaxine is continued postnatal, as demonstrated by data showing a significant difference between the DC and DD ND+35 groups. This trend is also seen in the FRL ND+60 group, where the same phenomenon is seen, suggesting that this effect lasts into early adulthood. However, the effect with the prenatal venlafaxine treatment is not seen in late adulthood at ND+90, suggesting that the original prenatal venlafaxine-induced increase in depressive-like behaviour in FRL rats is reversed in later adulthood at ND+90.

The enhanced immobility in FRL rats in the DC treatment group was not replicated in FSL rats, suggesting a role for genetic susceptibility. In the FSL ND+60 group there were significant reductions in immobility in all venlafaxine treatment groups (i.e. CD, DC & DD). These data suggest that early-life venlafaxine administration reverses the inherent depressive-like effect of FSL rats, to reach similar levels seen with drug naïve FRL rats. This was not observed at ND+35 and demonstrates a remarkable long-lasting effect manifesting only in early adulthood. This effect is only seen in stress-sensitive rats, suggesting a prominent role of genetic susceptibility.

However, in FSL rats at ND+90 the immobility is increased in DC and DD treatment groups, suggesting that the beneficial venlafaxine-induced effects seen at ND+60 are now reversed. These data also further supports the role of genetic susceptibility in the response of rats.

Due to the weak statistical power, the multiple comparisons did not yield good results. After re-evaluating the data it was found that by comparing only the CC to the DD groups using a Student's t-test that there is significance between these two groups. These comparisons will be discussed below.

Figure 4-5 shows the immobile behaviour of the tested rats before and after venlafaxine in the FST at the specified ages.



**Figure 4-5:** Immobile behaviour shown by the FRL and FSL rats in the FST on the different specified ages (a, b, c and d) following vehicle and venlafaxine treatment. The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ) and statistical significance taken as  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*).

It can be seen in Figure 4-5 a & c that early-life administration of venlafaxine to FRL rats does not change immobility behaviour in adulthood at ND+60 or ND+90. However, in stress-sensitive FSL rats, early-life administration of venlafaxine respectively decreases and increases immobility at ND+60 and ND+90, as compared to vehicle-treated animals. These effects imply that early-life administration of venlafaxine to stress-sensitive animals leads to long-lasting antidepressant-like effects at ND+60, but depressogenic-like effects at ND+90.

It should be noted, however, that the vehicle-treated FSL rats at ND+90 did not display enhanced immobility relative to the corresponding FRL rats, suggesting that the depressive-like responses of the stress-sensitive rat line was lost by this age. Previous data (Overstreet et al., 2005; Overstreet, 1993) indicated that even older FSL rats still display enhanced immobility and our

data can only be explained by additional stress during the acclimatisation of the rats following the renovations of the animal centre (NWU Vivarium – as explained before). However, that early-life venlafaxine treatment enhanced immobility in FSL rats is still significant, relating to a real pharmacological effect.

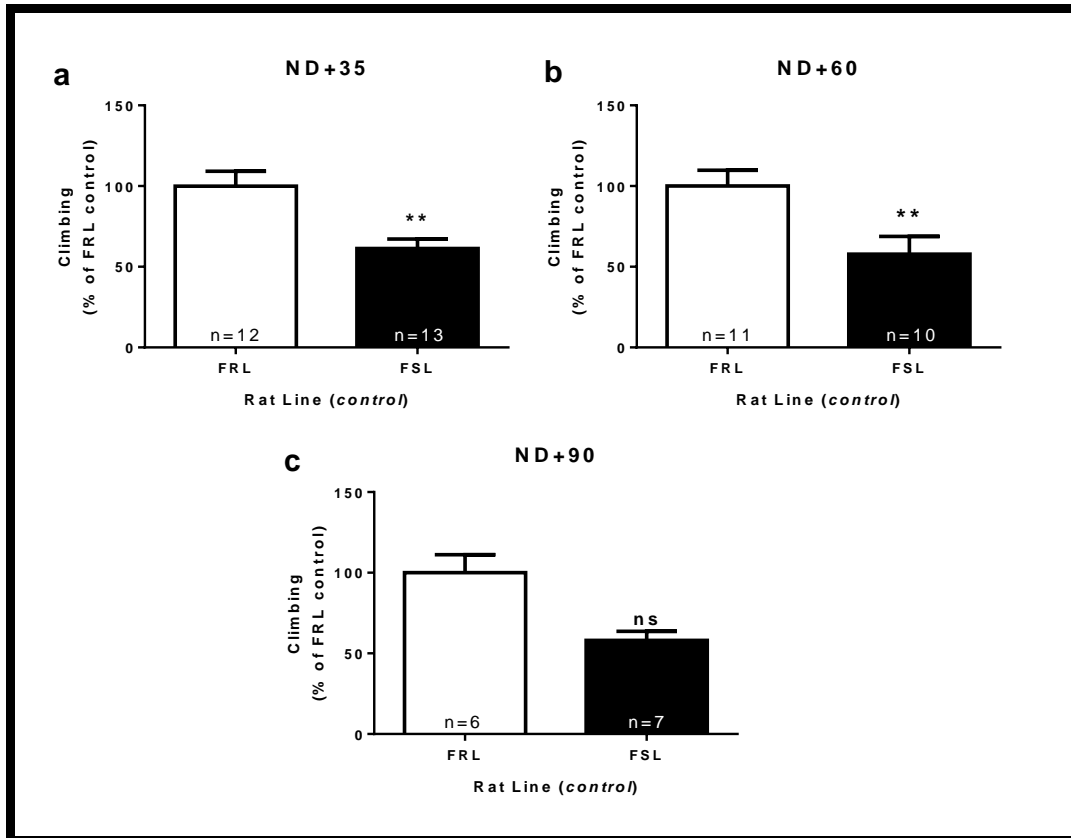
Acute drug effects cannot account for any of the effects in Figure 4-5, since the last dose of venlafaxine was administered on ND+17, 43 days before the ND+60 test and 73 days before the ND+90 test. It is therefore likely that these effects result from neurodevelopmental or long-lasting neuromodulatory effects of early-life administration of venlafaxine. It has been shown in a number of studies that the brain's synaptic development is influenced by changing levels of DA (Gelbard et al., 1990; Kalsbeek et al., 1988; Lankford et al., 1988; Todd, 1992), NE (Feeney & Westerberg, 1990; Kline et al., 1994) and 5-HT (Kuppermann & Kasamatsu, 1984; Lauder & Krebs, 1978; Whitaker-Azmitia & Azmitia, 1986) during early-life. Being exposed to drugs that affect the levels of these neurotransmitters at a young age may cause changes in the development of the brain and these effects will only manifest later in life (Andersen & Navalta, 2004).

#### **4.2.2 Climbing behaviour**

In light of the demonstrated altered immobility behaviour in FSL rats following early-life exposure to venlafaxine, it was important to investigate whether these effects can be associated with altered noradrenergic neurotransmission. Climbing behaviour in the FST is associated with noradrenergic activity, where increased climbing relates to enhanced noradrenergic neurotransmission (Cryan et al., 2002).

##### **4.2.2.1 FRL control vs. FSL control**

It was important to understand whether drug-naïve FSL and FRL rats displayed similar or different depressive-like behaviour. Therefore we determined the antidepressive-like behaviour as a percentage of the time spent climbing in the FST by the FRL CC and FSL CC groups, as depicted in Figure 4-6.

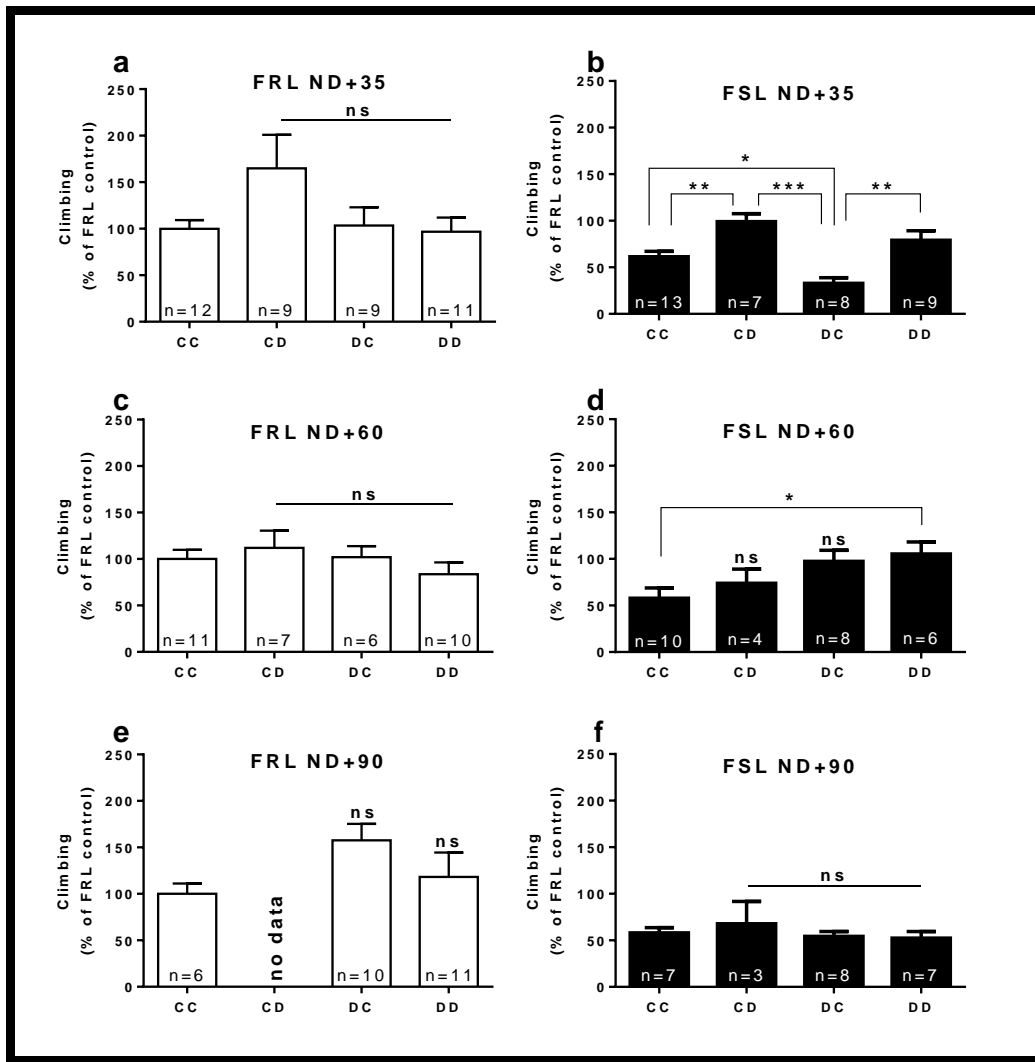


**Figure 4-6** Percentage time spent climbing in the FST by FRL and FSL control groups at postnatal day 35 (a), 60 (b) and 90 (c). The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ) and \*\* = significant (i.e.  $p < 0.01$ ).

Figure 4-6 shows that drug-naïve FSL rats showed a decrease in climbing activity in the forced swim test at ND+35 and ND+60. As climbing behaviour is used as a measure of noradrenergic neurotransmission, the data suggest that the increase in immobility of the FSL rat is related to a decrease in noradrenergic neurotransmission. There is however no significant difference with regard to the climbing behaviour shown by the ND+90 group, which correlates to the behaviour shown where the ND+90 FSL group had the same immobility as the ND+90 FRL group.

#### 4.2.2.2 Effects of early-life venlafaxine administration

The effect of pre- and/or postnatal venlafaxine on climbing behaviour is shown in Figure 4-7:



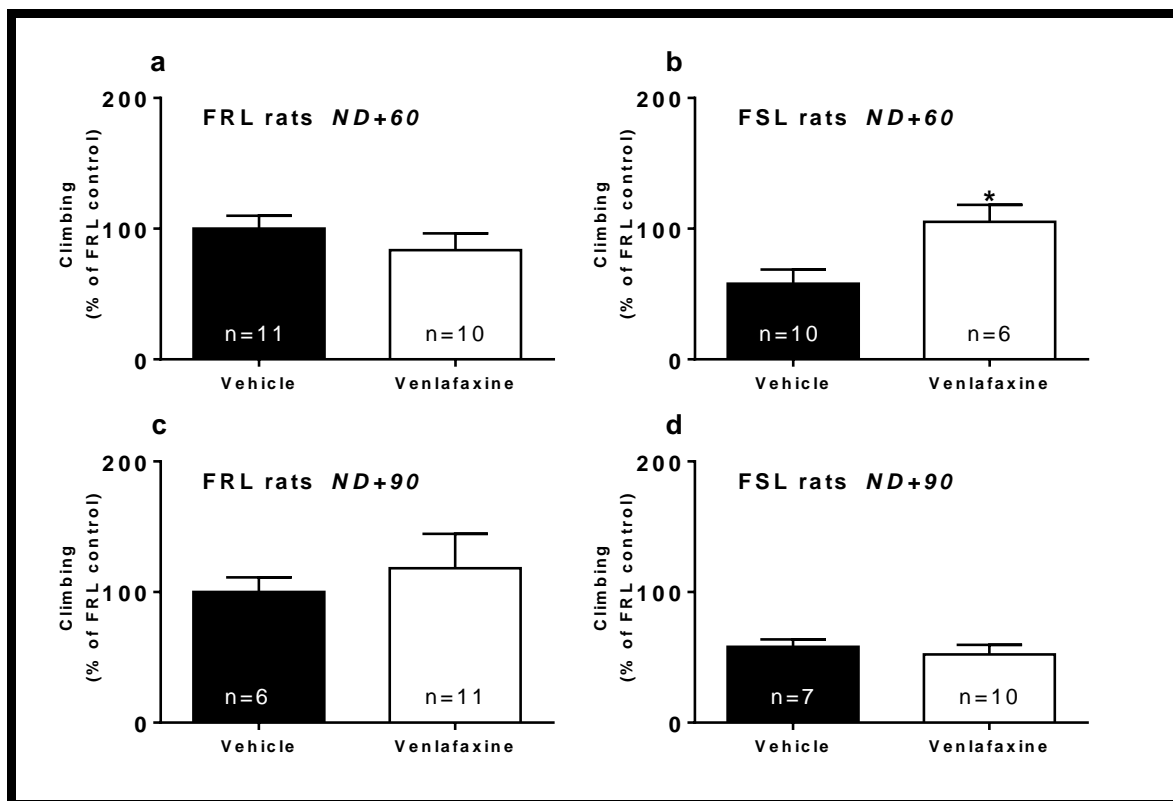
**Figure 4-7:** Climbing behaviour shown in the forced swim test by FRL and FSL venlafaxine treated rats at postnatal day 35 (a, and b), 60 (c, and d) and 90 (e, and f). The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ) and \*\* = significant (i.e.  $p < 0.01$ ).

It can be seen in Figure 4-7 that no significant venlafaxine-induced changes were found in FRL rats at ND+35, ND+60 or ND+90. However, significant venlafaxine-induced changes in climbing behaviour were seen with FSL rats at ND+35 and 60. At ND+35 it suggests that only prenatal treatment with venlafaxine (DC) lowers climbing behaviour significantly. This, however, is reversed if the treatment is given pre- and postnatal (DD). The data also suggests that postnatal venlafaxine treatment (CD) increases climbing behaviour in the FSL ND+35 group. In the FSL ND+60 group there is a

significant increase in climbing behaviour shown after pre- and postnatal early-life treatment with venlafaxine (DD). This suggests that the decrease in immobile behaviour seen in this group is due to pro-noradrenergic mechanisms. There were no significant venlafaxine-induced changes in climbing behaviour of the FSL rats at ND+90s.

As explained earlier (§4.2.1.2), single comparisons of the climbing behaviour will now be discussed.

Figure 4-8 depicts the climbing behaviour of FRL and FSL CC and DD rats at the indicated ages in the FST, following early-life administration of venlafaxine or vehicle control.



**Figure 4-8:** Climbing behaviour shown by FRL and FSL rats in the FST on the different specified ages (a, b, c and d) following vehicle and venlafaxine treatment. The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ) and statistical significance taken as  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*).

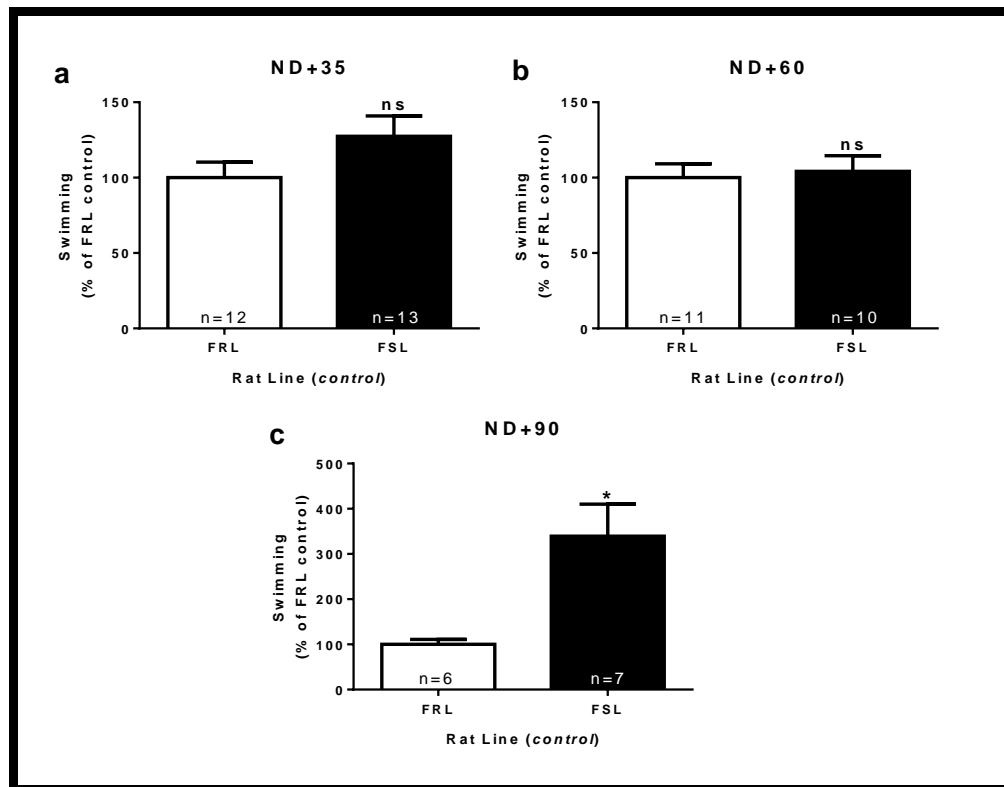
The data in Figure 4-8 suggests that early-life treatment with venlafaxine has no significant effect on climbing behaviour in FRL rats at ND+60 or ND+90, or in FSL rats at ND+90. However, in FSL rats at ND+60, there is a marked increase in climbing behaviour, suggesting that the early-life venlafaxine-induced antidepressive-like behaviour seen in FSL rats at this age may be related to enhanced noradrenergic neurotransmission. It has been suggested in other studies that treatment with a dual NE/5-HT reuptake inhibitor (milnacipran) has an increasing effect on climbing behaviour in Sprague-Dawley rats (Rénéric et al., 2001; Rénéric et al., 2002).

### **4.2.3 Swimming behaviour**

In light of the demonstrated altered immobility behaviour in FSL rats following early-life exposure to venlafaxine, as with climbing behaviour above, it was important to investigate whether these effects can be associated with altered serotonergic neurotransmission. Swimming behaviour in the FST is associated with serotonergic activity, where increased swimming relates to enhanced serotonergic neurotransmission (Cryan et al., 2002).

#### **4.2.3.1 FRL control vs. FSL control**

It was important to understand whether drug-naïve FSL and FRL rats displayed similar or different depressive-like behaviour. Therefore we determined the antidepressive-like behaviour as a percentage of the time spent swimming in the FST by the FRL CC and FSL CC groups, as depicted in Figure 4-9.

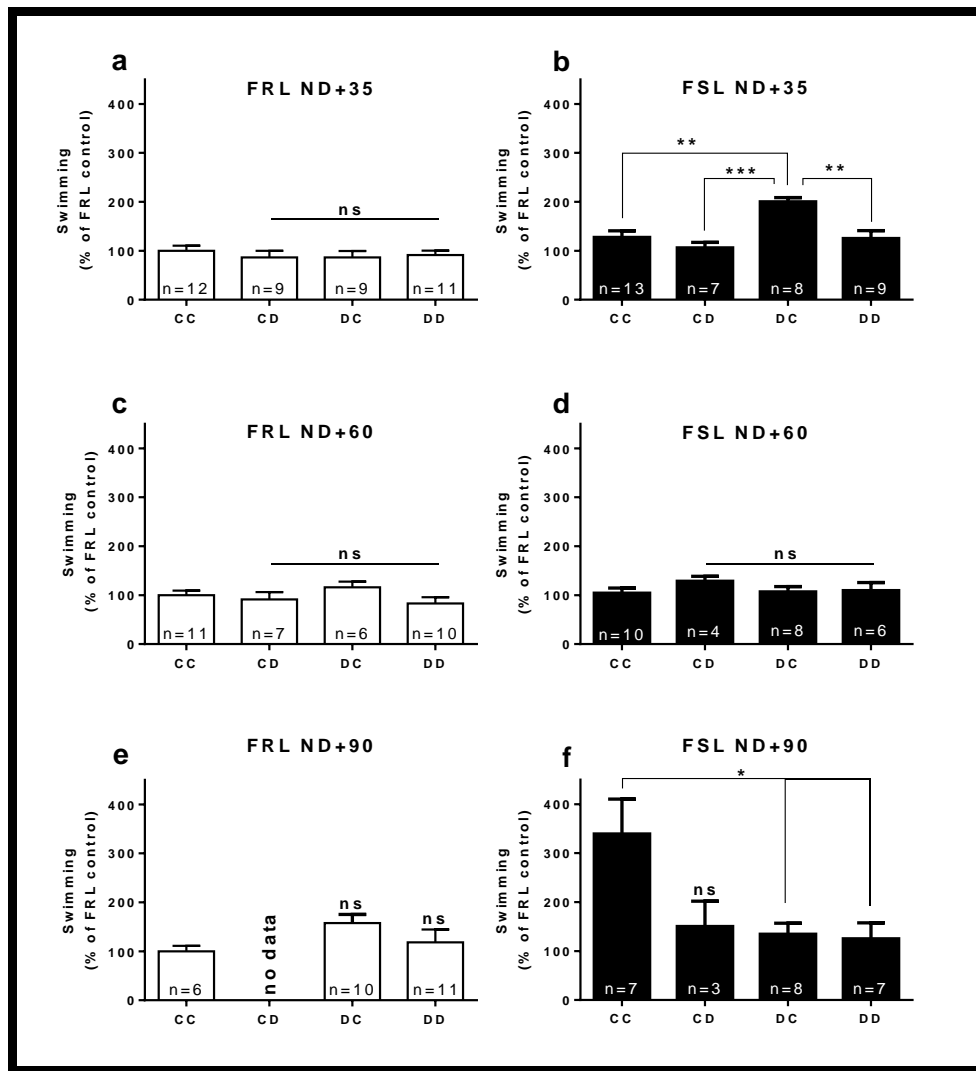


**Figure 4-9** Percentage time spent swimming in the FST by FRL and FSL control groups at postnatal day 35 (a), 60 (b) and 90 (c). The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ).

The data depicted in Figure 4-9 suggests that there were no significant changes in the swimming behaviour shown by the ND+35 and ND+60 FRL and FSL groups in the FST. There was however an increase in swimming behaviour shown by the FSL rats at ND+90 when compared to the corresponding FRL group. This could suggest that the decrease in immobile behaviour shown in the FSL ND+90 group is caused by an increase in serotonergic neurotransmission, as swimming behaviour is indicative of serotonergic neurotransmission.

#### 4.2.3.2 Effects of early-life venlafaxine administration

The effect of treatment with venlafaxine pre- and/or postnatal is shown in Figure 4-10:



**Figure 4-10:** Swimming behaviour shown in the FST by FRL and FSL venlafaxine treated rats at postnatal day 35 (a, and b), 60 (c, and d) and 90 (e, and f). The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ).

It can be seen in Figure 4-10 that no significant venlafaxine-induced changes were found in FRL rats at ND+35, ND+60 or ND+90. However, the swimming behaviour of the FSL at ND+35 changed with both pre- and/or postnatal early-life treatment with venlafaxine. This data suggests that prenatal venlafaxine increases swimming behaviour shown by these rats, which corresponds to the decrease in climbing behaviour shown by the same group (Figure 4-7) during the FST.

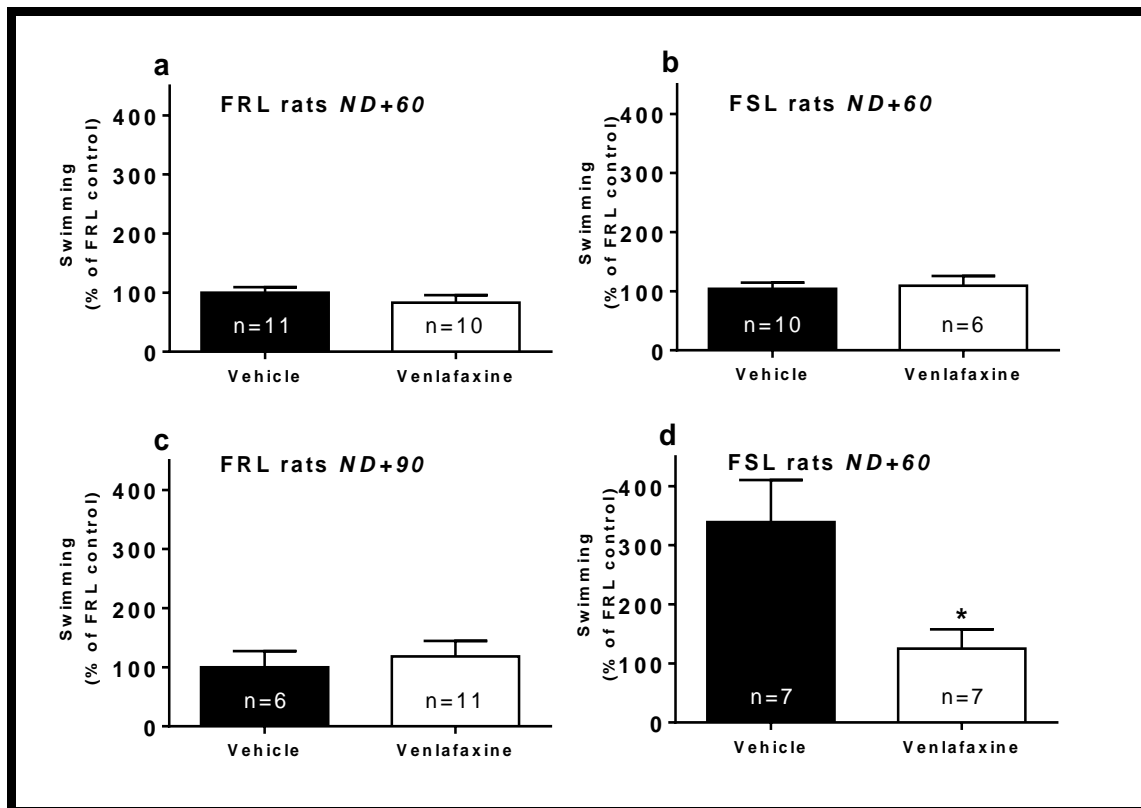
It was shown in Figure 4-4 that immobility was not altered by early-life venlafaxine in FSL rats at ND+35. However, we have now seen in Figure 4-7 and Figure 4-10 that FSL rats at ND+35 exhibit changes in both climbing and swimming behaviour, suggesting that, even though the different treatment regimens do not affect the animals' depressive-like behaviour, it does seem to have an effect on noradrenergic and serotonergic neurotransmission.

In Figure 4-10f there is a statistically significant decrease in swimming behaviour of FSL at ND+90, in the DC and DD groups. The decrease in swimming behaviour in the CD group did not reach statistical significance, most likely due to a lack of statistical power of the data. These data suggest that the increase in immobile behaviour exhibited by this group in the FST (Figure 4-4d) is mediated by reduced serotonergic neurotransmission.

In summary, the data collected from the FST suggest that early-life administration of venlafaxine may change behaviour later in life differently in stress sensitive and control rats. This supports a role for genetic susceptibility later in life in response to early-life administration of venlafaxine. In a previous study it was reported that chronic treatment of Sprague Dawley rats with venlafaxine significantly increased swimming behaviour in the animals, without affecting climbing behaviour (Larsen et al., 2010). It is important, however, to note that in the current study we employed a different treatment regimen and tested at different ages.

As explained earlier (§4.2.1.2), single comparisons of the swimming behaviour will now be discussed.

Figure 4-11 depicts the swimming behaviour in the FST at the specified ages, following early-life administration of venlafaxine or vehicle control.



**Figure 4-11:** Swimming behaviour shown by FRL and FSL rats in the FST on the different specified ages following vehicle and venlafaxine treatment. The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ) and statistical significance taken as  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*).

The data in Figure 4-11 suggests that early-life treatment with venlafaxine has no significant effect on swimming behaviour in FRL rats at ND+60 or ND+90, or in FSL rats at ND+90. However, in FSL rats at ND+90, there is a marked decrease in swimming behaviour, suggesting that the early-life venlafaxine-induced depressogenic-like behaviour seen in FSL rats at this age may be related to impaired serotonergic neurotransmission.

From the data it can be observed that there is a change occurring between ND+60 and ND+90 with regard to climbing and swimming behaviour (Figure 4-8 & Figure 4-11), respectively. At ND+60 there was a venlafaxine-induced increase in climbing behaviour in the FSL rat that was nullified at ND+90. On the other hand there was no effect on the swimming behaviour on ND+60 and a decrease in swimming activity at ND+90. These observations suggest that

the antidepressive-like effect of venlafaxine at ND+60 after chronic early-life treatment (Figure 4-5) is related to increased noradrenergic neurotransmission (Figure 4-8). Also, the depressogenic-like effect at ND+90 (Figure 4-5) is related to decreased serotonergic neurotransmission (Figure 4-11). In a previous study, however, it was found that venlafaxine treatment of Sprague Dawley rats increased both climbing and swimming behaviour in the FST (Reneric & Lucki, 1998). The difference in observations between the current study and the study by Reneric and Lucki (1998) could be explained by the use of different rat lines as well as the different drug treatment regimens used.

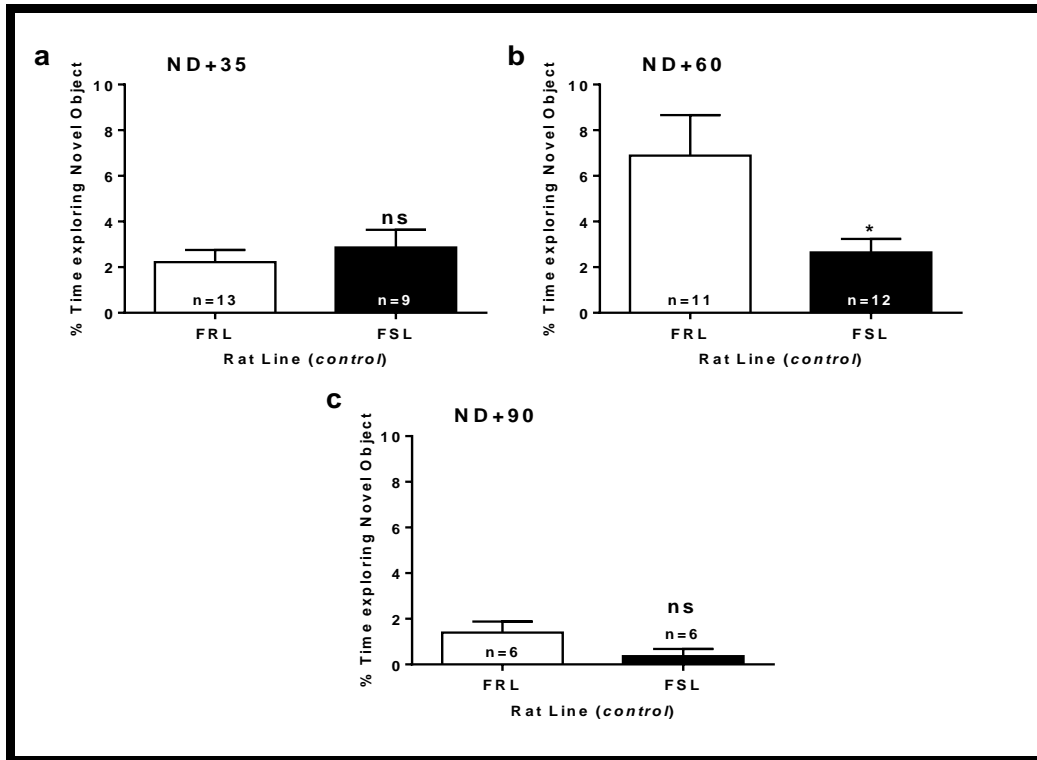
The most important finding from this study is that early-life treatment with venlafaxine may reverse congenital tendency for depressive-like behaviour in FSL rats during early adulthood (ND+60) via pro-noradrenergic mechanisms, but increase the risk of depressive-like behaviour at ND+90 via anti-serotonergic mechanisms.

### **4.3 Novel Object Recognition Test**

Memory and cognition was tested in the NORT as explained in Chapter 3. Results were determined as a percentage of the time spent exploring the novel object.

#### **4.3.1 FRL control vs. FSL control**

It was of importance to understand whether drug-naïve FSL and FRL rats displayed similar or different levels of cognition and memory consolidation. Therefore we determined novel object recognition as a percentage of the time spent exploring the novel object in the retention trial by the FRL CC and FSL CC groups, as depicted in Figure 4-12.

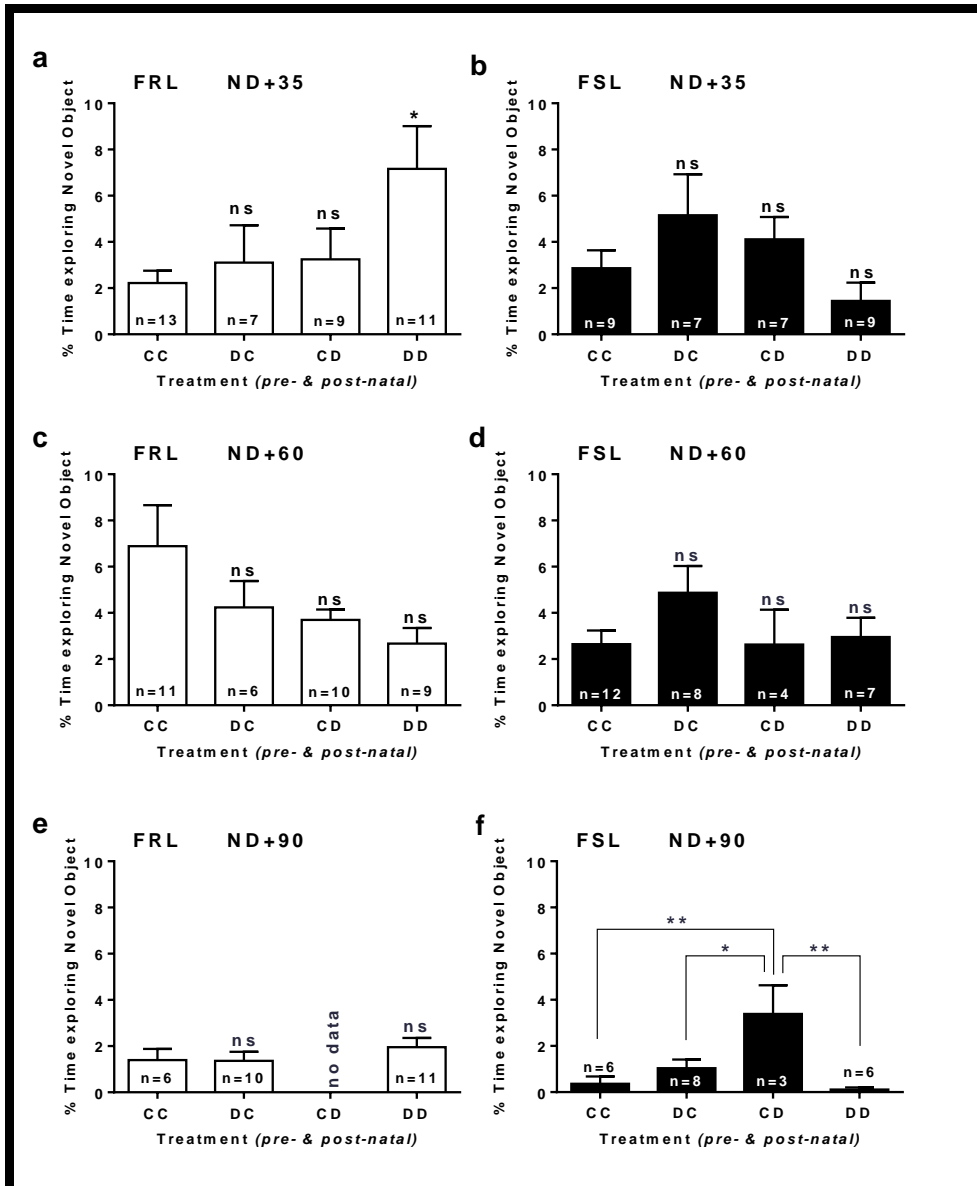


**Figure 4-12:** Percentage time spent exploring the novel object during the retention trial of the NORT by FRL and FSL control groups on postnatal day 35 (a), 60 (b) and 90 (c). The number of data points per treatment group is indicated on the graph bars. The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ).

The data in Figure 4-12 suggests that there is no significant change in the ND+35 and 90 groups between the FRL and FSL control groups. There is however a significant decrease at ND+60 in the cognitive ability of FSL control rats compared to FRL controls. This data suggests that FSL control rats exhibit impaired memory consolidation relative to FRL control rats in early adulthood at ND+60.

#### 4.3.2 Effects of early-life venlafaxine administration

The effects of the different venlafaxine regimens on cognition and impairment of memory were determined in the NORT and depicted in Figure 4-13. It is expressed as a percentage of the time spent exploring the novel object in the retention trial.



**Figure 4-13:** Percentage time spent exploring the novel object during the retention trial of the NORT by FRL and FSL rats following venlafaxine treatment during the indicated early-life phases. FRL and FSL venlafaxine treated groups during specified early-life phases at postnatal day 35 (a, and b), 60 (c, and d) and 90 (e, and f). The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ) and \*\* = significant (i.e.  $p < 0.01$ ).

As can be seen in Figure 4-13, exploration of the novel object is increased by venlafaxine in the DD treatment group in FRL rats at ND+35. The same trend is not seen in FSL rats, where no statistically significant differences were observed at ND+35. No statistically significant differences could be

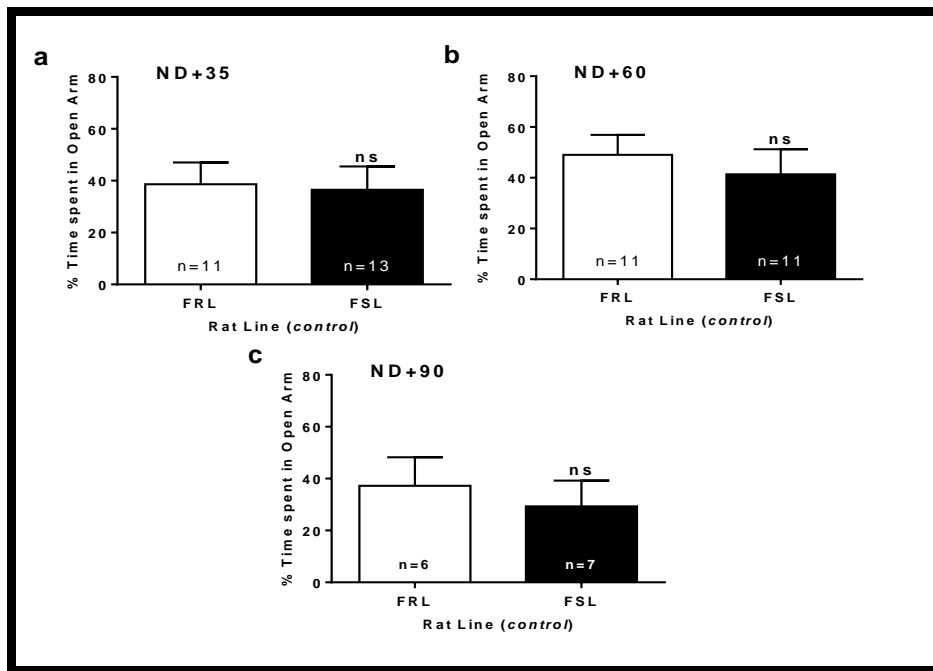
demonstrated at ND+60 in either FRL or FSL rats, which may relate to the lack of statistical power of the data. However, at ND+90, statistically significant increases in the CD treatment group of FSL rats were different from all other treatment groups. The results suggest that early-life treatment may variably affect cognition later in life and that such effects may be dependent on genetic susceptibility and age. Due to the small number of animals used, this requires further investigation.

#### **4.4 Elevated Plus Maze**

The elevated plus maze is used as a measure of anxiety-like behaviour in rats. Anxiety-like behaviour is estimated by the rats' tendency to either explore the open arms (reduced anxiety), or to stay in the closed arms (enhanced anxiety) as explained in Chapter 3

##### **4.4.1 FRL control vs. FSL control**

It was important to understand whether drug-naïve FSL and FRL rats displayed similar or different anxiety-like behaviour. Therefore we determined the anxiety-like behaviour as a percentage of the time spent in the open arms by the FRL CC and FSL CC groups, as depicted in Figure 4-14.

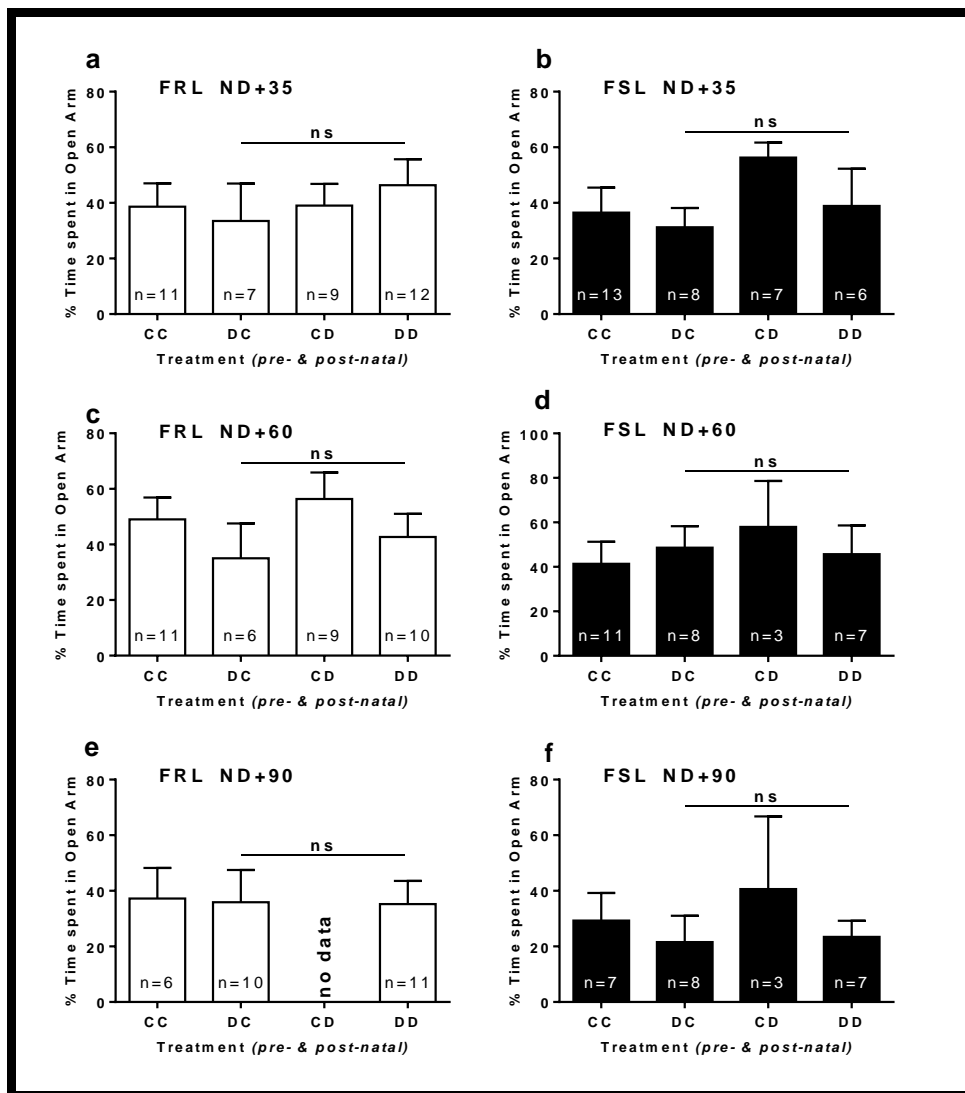


**Figure 4-14:** Percentage time spent in the open arm of the elevated plus maze by FRL and FSL control groups at postnatal day 35 (a), 60 (b) and 90 (c). The number of data points per treatment group is indicated on the graph bars. Statistical analysis included the Student's t test, with ns = not significant (i.e.  $p > 0.05$ ).

The data in Figure 4-14 suggests that there are no significant differences in anxiety-like behaviour between FRL and FSL rats at any age group. Several studies have suggested that increased anxiety-like behaviour is not a prominent characteristic of the FSL rat under normal conditions (Neumann et al., 2011; Overstreet et al., 2005). These results could therefore have been expected. FSL rats, however, seem to exhibit more social stress than FRL rats (Overstreet et al., 2005), so that such testing (not performed in the current study) could have revealed differences.

#### 4.4.2 Effects of early-life venlafaxine administration

Figure 4-15 depicts the effects of the different treatment regimens on the anxiety-like behaviour of the animals as determined in the elevated plus maze over a five minute trial. Results were calculated as percentage of the time spent in the open arm.



**Figure 4-15:** Percentage time spent in the open arm of the elevated plus maze test by FRL and FSL rats after the specified venlafaxine treatment at postnatal day 35 (a, and b), 60 (c, and d) and 90 (e, and f). The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ).

Statistical analyses of the data in Figure 4-15 did not reveal any statistically significant differences in the time spent in the open arms (i.e. anxiety-like behaviour) between any of the treatment groups. Although differences cannot be excluded due to the statistical weakness of the data, these results are not entirely unexpected and most likely a true reflection of the effects of drug treatment. Reports on the effect of chronic venlafaxine treatment on anxiety-like behaviour are inconclusive, as several studies found no changes in

anxiety-like behaviour (de Oliveira et al., 2004; Larsen et al., 2010). In contrast to these studies, other studies have demonstrated a significant increase following treatment with a selective serotonin reuptake inhibitor and a tricyclic antidepressant (Kokras et al., 2011; Slabbert, 2010). Several studies have suggested that increased anxiety-like behaviour is not a prominent characteristic of the FSL rat under normal conditions (Neumann et al., 2011; Overstreet et al., 2005). According to Overstreet and Griebel (2004) the more sensitive test to assess anxiety in the FSL rat is the social interaction test. In this test, FSL rats spend notably less time interacting with each other when compared to the FRL rats (Overstreet & Griebel, 2004). It is important to note that in these studies the treatment regimen as well as the age of testing differed, possibly explaining the differences in the results that were obtained.

#### **4.5 Neurobiological concentrations of I-NE and 5-HT**

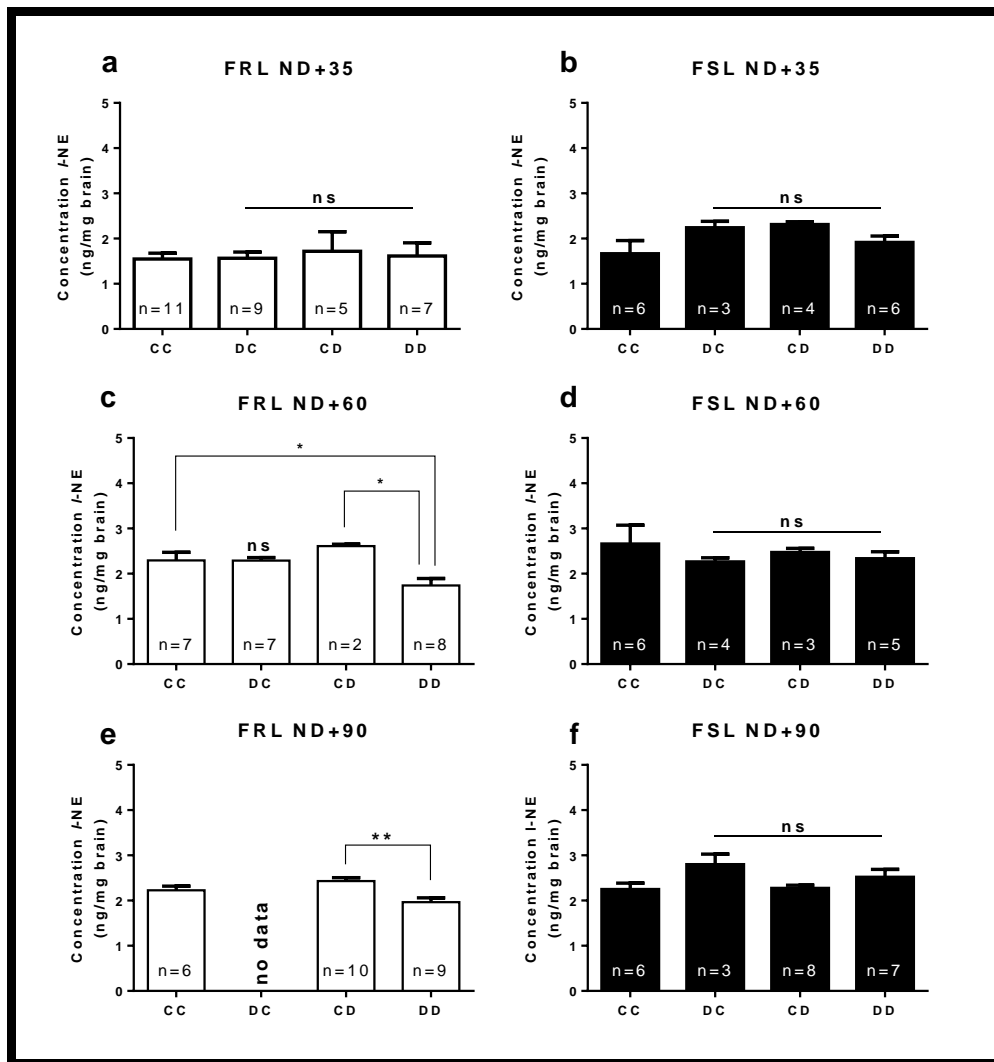
Noting the early-life venlafaxine-induced changes in monoaminergic neurotransmission from the climbing and swimming behaviour shown in the data above, the next logical step was to investigate biomarkers of such changes. Since venlafaxine is a dual NA and 5-HT reuptake inhibitor, we opted to analyse these two neurotransmitters specifically, which would provide more specific information on its probable mode of action in FSL and FRL rats. The concentrations of I-norepinephrine (I-NE) and serotonin (5-HT) were determined in the prefrontal cortex and hippocampus of treated rats using high pressure liquid chromatography with electrochemical detection (HPLC-ECD), as explained in Chapter 3. Due to small n numbers leading to insufficient statistical power, the data were also analysed using the Cohen test for effect size to evaluate practical significance of any differences between data sets.

##### **4.5.1 Concentrations of I-NE and 5-HT in the rat brain**

Concentrations of I-NE and 5-HT were determined in the prefrontal cortex and hippocampus of treated rats using HPLC-ECD as explained in Chapter 3.

###### **4.5.1.1 Concentration of I-NE in the prefrontal cortex**

In Figure 4-16 the results of the determination of I-NE concentrations in the prefrontal cortex across the different treatment groups are given.

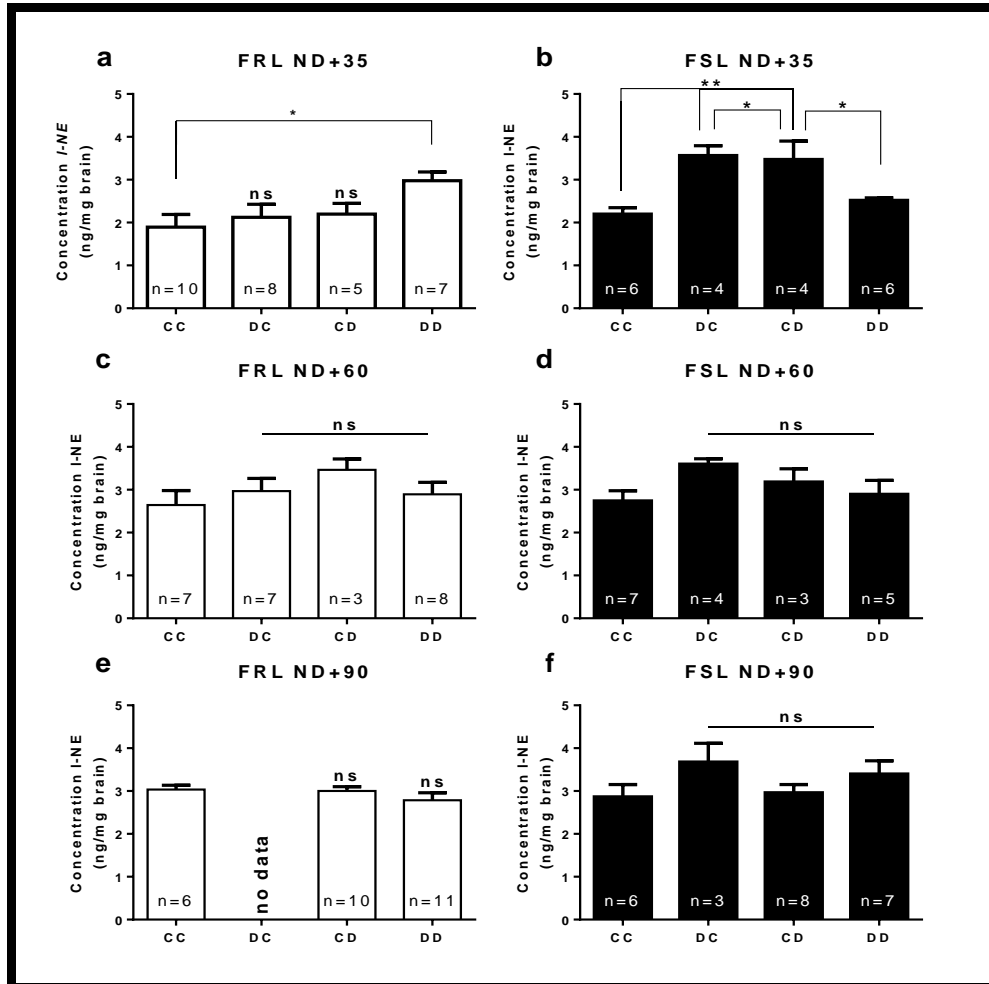


**Figure 4-16:** Concentration of I-NE in the prefrontal cortex after early-life treatment with venlafaxine in FSL and FRL rats, at the specified ages in later life. The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ) and \*\* = significant (i.e.  $p < 0.01$ ).

As can be seen in Figure 4-16, early-life venlafaxine did not change I-NE levels in the prefrontal cortex in FRL rats at ND+35 and in FSL rats at ND+35, ND+60 and ND+90. However, in FRL rats at ND+60 and ND+90 select changes were observed. As discussed in later in this section, these subtle changes in the I-NE levels could relate to indirect serotonin-mediated effects of venlafaxine via interaction with 5-HT<sub>2C</sub> receptors.

#### 4.5.1.2 Concentration of I-NE in the hippocampus

Figure 4-17 shows the levels of I-NE in the hippocampus across the different treatment groups and at the specified ages.



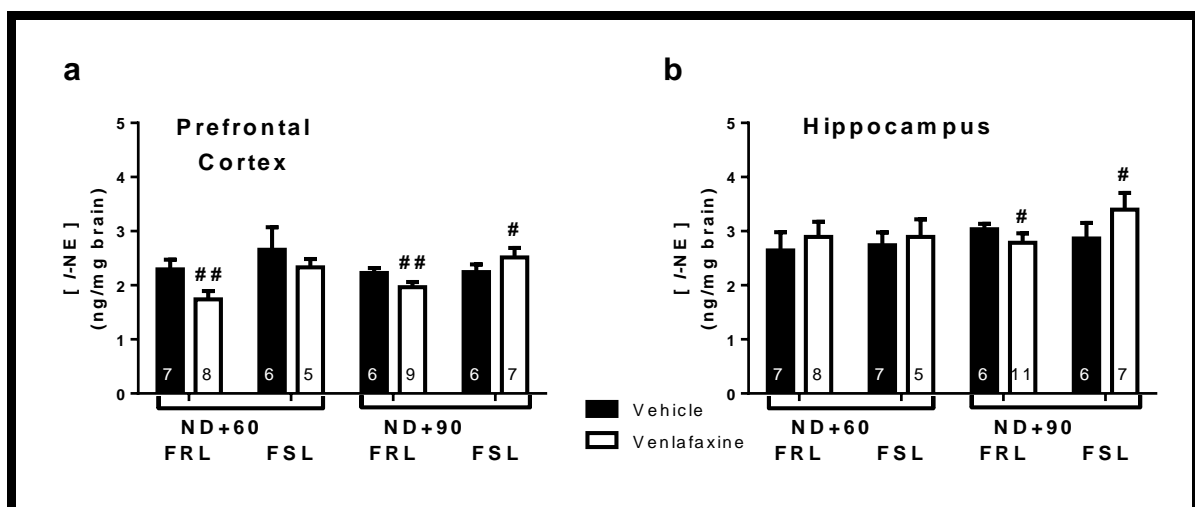
**Figure 4-17:** Concentration of I-NE in the hippocampus after early-life treatment with venlafaxine in FSL and FRL rats, at the specified ages in later life. The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ) and \*\* = significant (i.e.  $p < 0.01$ ).

The data depicted in Figure 4-17 show that there is a significant increase in the concentration of I-NE in the FRL rats during adolescence at ND+35 after pre- and postnatal treatment with venlafaxine (DD). In the FSL ND+35 group the data shows a significant increase in I-NE when venlafaxine was administered pre- or postnatal. This increase is reversed if venlafaxine is given

both pre-and postnatal. The data corresponds to climbing behaviour demonstrated by these animals in the FST, where an increase in climbing behaviour following postnatal venlafaxine administration. It is interesting to note that prenatal venlafaxine increased I-NE concentration at ND+35 in the hippocampus in FSL rats, but the rats showed a decrease in climbing behaviour in the FST. This could be because of receptor desensitization or down regulation caused by prenatal venlafaxine treatment and warrants further investigation.

As explained earlier (§4.2.1.2), single comparisons of the I-NE concentrations will now be discussed.

Figure 4-18 depicts the total tissue concentrations of I-NE in respectively the prefrontal cortex and hippocampus of FRL and FSL rats at ND+60 and ND+90, following early-life administration of venlafaxine or vehicle control.



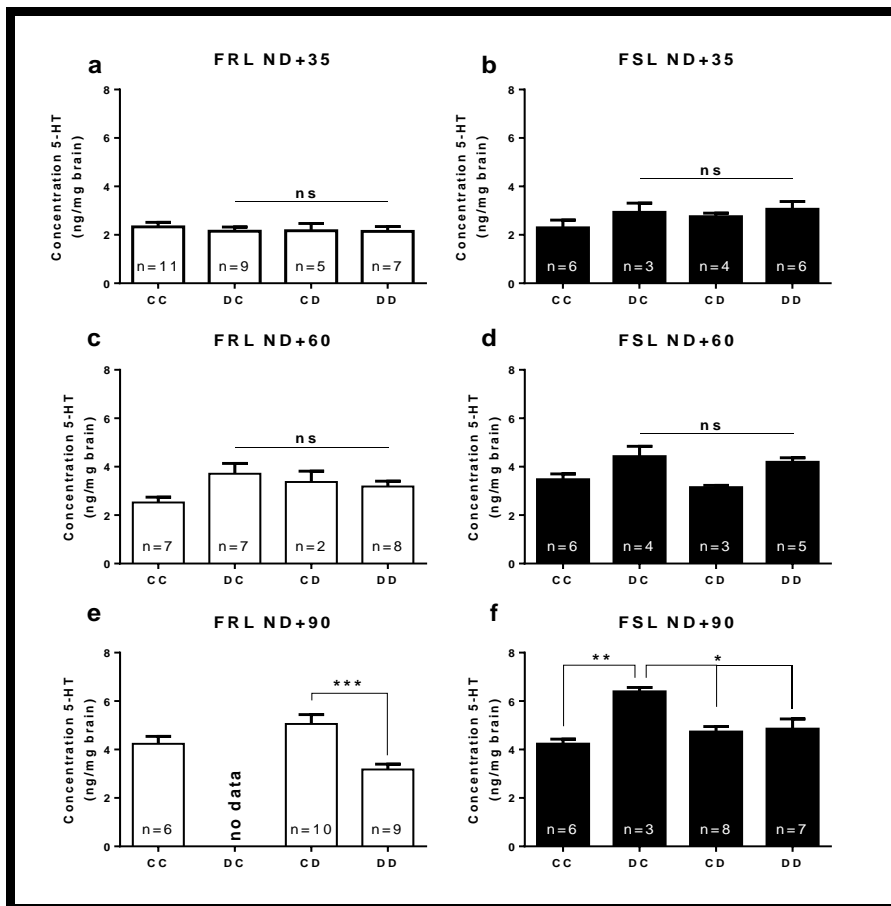
**Figure 4-18:** Concentration of I-NE in the prefrontal cortex, a), and hippocampus, b), of vehicle treated and venlafaxine treated FSL and FRL rats at the different specified ages. Data were analysed to show practical significance with the Cohen test and significance was taken at  $d > 0.5$  (#) and  $d > 0.8$  (##).

It can be seen in Figure 4-18 that, although there was no statistically significant differences between I-NE levels in the various treatment groups, there were a number of practically significant differences. An interesting trend can be observed in cases where practically significant differences could be

demonstrated. In all of these instances early-life administration of venlafaxine reduced I-NE levels in FRL rats, and increased I-NE levels in stress sensitive FSL rats. However, these data do not correspond with the enhanced climbing behaviour (noradrenergic activity) at ND+60 described earlier in the FST results. This discrepancy between the behaviour and the I-NE levels can be explained by previous studies that have found that 5-HT modifies noradrenergic transmission (Hadjjeri et al., 1997; Millan et al., 2000), and as we will see, venlafaxine increased 5-HT activity (see Fig 4-6). There are also reports that the stimulation of the 5-HT<sub>2C</sub> receptor decreases the release of NE in the frontal cortex (Millan et al., 1998). We therefore suggest that the treatment with venlafaxine could have increased the serotonin-mediated stimulation of the 5-HT<sub>2C</sub> receptor via inhibition of serotonin reuptake, resulting in a decrease in the I-NE levels.

#### **4.5.1.3 Concentration of 5-HT in the prefrontal cortex**

Figure 4-19 depicts the 5-HT concentrations in the prefrontal cortex across the different treatment groups and at the specified ages.

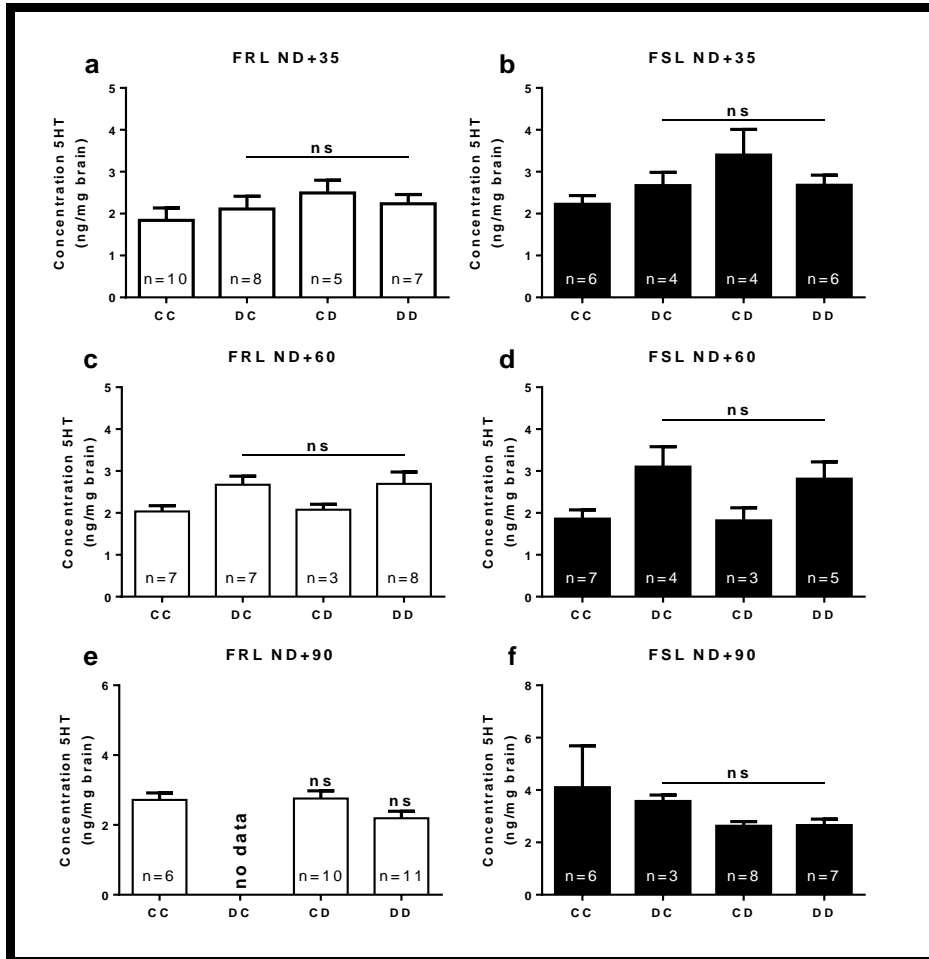


**Figure 4-19:** Concentration of 5-HT in the prefrontal cortex after early-life treatment with venlafaxine in FSL and FRL rats, at the specified ages in later life. The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ) and \* = significant (i.e.  $p < 0.05$ ) and \*\* = significant (i.e.  $p < 0.01$ ).

As can be seen in Figure 4-19, early-life venlafaxine did not change 5-HT levels in the prefrontal cortex in FRL or FSL rats at ND+35 and ND+60. However, in FRL rats at ND+90 the trend towards increased 5-HT levels in the CD group and decreased 5-HT levels in the DD group was statistically significantly different from one another. In FSL rats at ND+90 an increase in 5-HT levels was seen only in the DC treatment group. The data does not correspond with the venlafaxine-induced decrease in serotonergic neurotransmission seen with FSL rats at ND+90 in the FST (Figure 4-4f). Serotonergic levels and behaviour therefore do not seem to be directly related.

#### 4.5.1.4 Concentration of 5-HT in the hippocampus

Figure 4-20 depicts the 5-HT concentrations in the hippocampus across the different treatment groups.

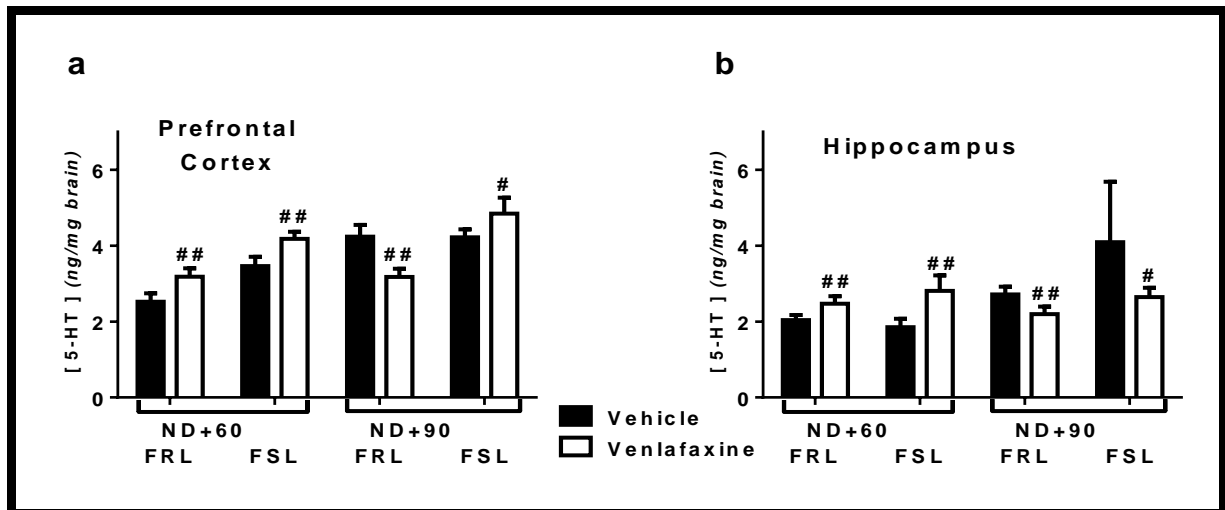


**Figure 4-20:** Concentration of 5-HT in the hippocampus after early-life treatment with venlafaxine in FSL and FRL rats, at the specified ages in later life. The number of data points per treatment group is indicated on the graph bars. Statistical analyses for multiple comparison included the one-way ANOVA with a Tukey post-hoc test, with ns = not significant (i.e.  $p > 0.05$ ).

As can be seen from Figure 4-20, there were no significant venlafaxine-induced changes in the concentration of 5-HT in the hippocampus in any of the groups tested in this study. The data suggests that there are no long-lasting effects of early-life venlafaxine on the 5-HT concentration in the hippocampus.

As explained earlier (§4.2.1.2), single comparisons of the 5-HT concentrations will now be discussed.

Figure 4-21 depicts the total tissue concentrations of serotonin (5-HT) in respectively the prefrontal cortex and hippocampus of FRL and FSL rats at ND+60 and ND+90, following early-life administration of venlafaxine or vehicle control.



**Figure 4-21:** Concentration of 5-HT in the prefrontal cortex, a), and hippocampus, b) of vehicle treated and venlafaxine treated FSL and FRL rats at the different specified ages. Data were analysed to show practical significance with the Cohen test and significance was taken at  $d > 0.5$  (#) and  $d > 0.8$  (##).

In Figure 4-21 groups, there were a number of practically significant differences and a trend can be seen that early-life venlafaxine increased serotonin levels at ND+60 in both FRL and FSL rats, in both the prefrontal cortex and hippocampus. At ND+90 the picture changes slightly. Now a trend can be seen for early-life venlafaxine to decrease serotonin levels in the FRL rat prefrontal cortex and hippocampus, whereas it increased in the prefrontal cortex in FSL rats but decreased in the hippocampus. The venlafaxine-induced decrease in serotonin levels in the hippocampus of FSL rats at ND+90 corresponds with the decreased swimming behaviour (i.e. decreased serotonergic neurotransmission) observed from swimming behaviour derived from the FST tests (Figure 4-11d) described before. It has been reported in several studies that serotonergic abnormalities are evident in FSL rats

(Overstreet et al., 1994; Wallis et al., 1988; Zangen et al., 1997). One of these abnormalities is that FSL rats are more sensitive to the hypothermic effects of selective 5-HT<sub>1A</sub> receptor agonists (Overstreet et al., 1994; Wallis et al., 1988). Another abnormality is that FSL rats have been shown to have higher concentrations of 5-HT in the limbic areas of the brain when compared to the FRL rats, and it is normalised after chronic antidepressant therapy (Zangen et al., 1997).

The data from the study suggests that there were practically significant changes in both the I-NE and 5-HT concentration in the prefrontal cortex and hippocampus for almost all of the treatment groups. There is however no overall direct correlation with the swimming behaviour shown by the animals in the FST and the changes in the concentrations of I-NE and 5-HT.

# Chapter 5 Conclusion

## **5 Conclusion**

This chapter will give a brief summary of the study results, followed by conclusions from the results and discussions in Chapter 4, and finally make recommendations for prospective studies and lastly pointing out the limitation of the current study. It will be clear how the study addressed the study objectives outlined in Chapter 1.

### **5.1 Summary of results**

The following table will give a concise overview of all results in Chapter 4. The most important changes were observed with respect to the behaviour of the rats after treatment with venlafaxine (Table 5-1).

**Table 5-1:** Changes in the measured bio-behavioural parameters of FSL and FRL rats measured at ND+35, ND+60 and ND+90, following early-life administration of venlafaxine.

Parameter	ND+35								ND+60								ND+90							
	FRL				FSL				FRL				FSL				FRL				FSL			
	CC	CD	DC	DD	CC	CD	DC	DD	CC	CD	DC	DD	CC	CD	DC	DD	CC	CD	DC	DD	CC	CD	DC	DD
Immobility	↔	↔	↑	↔	↔	↔	↔	↔	↔	↔	↑	↔	↔	↔	↓	↓	↔	—	↔	↔	↔	↔	↔	↔
Climbing	↔	↔	↔	↔	↔	↑	↓	↔	↔	↔	↔	↔	↔	↔	↔	↑	↔	—	↔	↔	↔	↔	↔	↔
Swimming	↔	↔	↔	↔	↔	↔	↑	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	—	↔	↔	↔	↔	↓	↓
Anxiety-like	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	—	↔	↔	↔	↔	↔	↔
Cognition	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	—	↔	↔	↔	↑	↔	↔
Locomotor	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	—	↔	↔	↔	↔	↔	↔
PFC I-NE	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓	↔	↔	↔	↔	↔	—	↔	↓	↔	↔	↔	↔
Hipp I-NE	↔	↔	↔	↑	↔	↑	↑	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	—	↔	↔	↔	↔	↔	↔
PFC 5-HT	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	—	↔	↓	↔	↔	↑	↔
Hipp 5-HT	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	—	↔	↔	↔	↔	↔	↔

## 5.2 Final Conclusions

The current study investigated the long-lasting effects of early-life venlafaxine administration in FSL and FRL rats on depressive-like and anxiety-like behaviour, cognition and brain monoamine concentrations as manifested in adolescence, early-adulthood and mid-adulthood. Factors that could influence these effects included:

- the genetic susceptibility of the rat line employed (normal FRL rats versus stress-sensitive FSL rats),
- early-life chronic treatment regimens, including the pre-natal + postnatal administrations as saline + saline, saline + venlafaxine, venlafaxine + saline, venlafaxine + venlafaxine, and
- the age at which effects were measured (ND+35, ND+60 or ND+90).

Despite experimental difficulties and consequent weaknesses of the experimental data, the current study provided a number of answers to the research questions. Although inconclusive in some instances, the study results clearly indicated a role for each of the factors listed above.

### 5.2.1 The genetic susceptibility of the rat line employed (FSL vs. FRL rats)

In the current study there were significant differences with respect to cognition and anxiety- and depressive-like behaviours of drug-naïve FSL and FRL rat lines. As a well-described, validated model of depression, the FSL rat line was expected to yield different results than the FRL rat line. Such differences have been described in various previous studies (Dremencov et al., 2004; Liebenberg et al., 2010; Overstreet & Russell, 1982). We confirmed that FSL rats display enhanced immobility relative to FRL rats in the FST at ND+35 and ND+60 under our experimental conditions. This response was expected in FSL rats, known to display enhanced depressive-like behaviour (enhanced immobility), as was reported in several previous studies (Dremencov et al., 2004; Liebenberg et al., 2010; Overstreet & Russell, 1982). There were, however, no discernable differences in the immobility of FSL and FRL rats at ND+90. We believe that this lack of enhanced immobility in FSL rats may be explained by the stress elicited by renovations at the NWU Vivarium, as described in §4.2.1.1.

The results of the current study also demonstrated significantly impaired memory consolidation in adult (ND+60) FSL rats, relative to FRL rats. The time spent exploring the novel object in the retention trial of the novel object recognition test (NORT) was significantly lower in the control groups when comparing the FSL to the FRL rats. This decrease in cognitive ability of FSL rats is also in keeping with the results of previous studies. Clinical evidence also suggests a decrease in memory and memory consolidation in depressed patients (Günther et al., 2004).

Anxiety-like behaviour, however, was not different between FSL and FRL rats in the current study. Different from the current study, previous studies indicated differences in the anxiety-like behaviour of FSL and FRL rats (Kokras et al., 2011; Liebenberg, 2009). Again, we believe that the stressful adjustment and housing conditions following the renovations of the NWU Vivarium may be the culprit, particularly in stress-sensitive animals. These varying external stressors during the study were not within our control. It has been suggested in several previous studies that anxiety-like behaviour is not a prominent characteristic of the FSL rat line (Neumann et al., 2011; Overstreet et al., 2005), so that the lack of an expected anxiety response may be anticipated to result from such adverse conditions. However, it should be mentioned that anxiogenic behaviour as it manifests in the social interaction test, measuring a different aspect of anxiety during interaction with another rat, has been described as a more prominent characteristic of FSL relative to FRL rats (Overstreet & Griebel, 2004). The current study did not implement the social interaction test.

The data from the current study suggest that the FSL rat line presents a suitable translational model for further studies to evaluate the effect of chronic early-life treatment with an antidepressant. Besides the stress-sensitivity, FSL rats also respond to chronic and not acute antidepressant treatment, similar to humans, adding to its predictive validity and usefulness in the current study.

### **5.2.2 Effects of chronic early-life treatment with venlafaxine on FSL and FRL rats at various ages**

The results obtained from the current study suggest that there is indeed an effect on cognition and anxiety- and depressive-like behaviour at various ages after pre- and/or postnatal treatment with venlafaxine. The most marked effects are seen in

early adulthood at ND+60 and these changes mostly do not continue into later-adulthood at ND+90. However, at ND+35 (onset of adolescence) none of the minor differences reached statistical significance.

No significant changes in locomotor activity were recorded in the Digiscan® animal activity monitor with any of the venlafaxine treatment regimens. This suggests that there are no treatment-induced locomotor defects in the animals and confirms that data in the FST are a direct result of psychomotor and not locomotor effects (Dawson & Tricklebank, 1995). It was reported in previous studies, however, that locomotor behaviour may increase after chronic treatment with venlafaxine (de Oliveira et al., 2004; Kumar et al., 2010), albeit using different treatment regimens than used in the current study.

Results from the NORT test suggested that cognition and memory consolidation in FRL rats was enhanced only at ND+35 following the DD treatment regimen, and enhanced in FSL rats only at ND+90 following the CD treatment regimen (Figure 4-13). This effect observed in FRL rats during adolescence was reversed later in life. In FSL rats the increase in memory consolidation was evident only later in adulthood, suggesting a difference in time-dependent responses of FRL and FSL rats.

The EPM showed no treatment-induced changes in anxiety-like behaviour in the FRL and in FSL rats. Reports on the effect of chronic venlafaxine treatment on anxiety-like behaviour are inconclusive, as several studies found no changes in anxiety-like behaviour (de Oliveira et al., 2004; Larsen et al., 2010). In contrast to these studies, a significant increase was observed following treatment with a SSRI and a TCA in other studies (Kokras et al., 2011; Slabbert, 2010). Several studies have suggested that increased anxiety-like behaviour is not a prominent characteristic of the FSL rat under normal conditions (Neumann et al., 2011; Overstreet et al., 2005).

In the FST early-life venlafaxine treatment caused an increase in immobility in FRL rats at ND+35. This increase in immobility was not accompanied by any significant changes in climbing or swimming behaviour, or in the concentrations of I-NE and 5-HT in the prefrontal cortex or hippocampus. The mechanism for the increase in immobility in this group is unknown and warrants further investigation.

In the FST at ND+60 (Figure 4-5), immobile behaviour in FSL rats was significantly decreased at ND+60 following chronic, early-life treatment with venlafaxine. This decrease in immobility was accompanied by an increase in climbing behaviour, suggesting that the decrease in long-term venlafaxine-induced antidepressant-like response at ND+60 was mediated by pro-noradrenergic mechanisms (§4.2.2). At ND+90, however, this effect was reversed and immobility was even increased. This effect was accompanied by a decrease in swimming behaviour, suggesting that the long-term venlafaxine-induced depressogenic-like effect at ND+90 is mediated by anti-serotonergic mechanisms (§4.2.3). It is also interesting to note that the data suggest that even though there was no significant change in immobility at ND+35, that there were significant changes in the climbing and swimming behaviour. This suggests that, even though there were no changes in depressive-like behaviour, the treatment regimen with venlafaxine did cause changes in the behaviour in this age group. The concentrations of I-NE and 5-HT (Figure 4-16; Figure 4-17; Figure 4-19; Figure 4-20) in the prefrontal cortex and hippocampus did not reveal any mechanistic explanation for the observed changes and further investigation into this phenomenon is warranted. Previous studies in rats showed that it is not clear whether the activity or expression of NET and SERT may have a positive correlation with an antidepressant-like response (Mitchell et al., 2013).

In conclusion, the chronic early-life treatment with venlafaxine caused a significant decrease in depressive-like behaviour via noradrenergic mechanisms in early adulthood at ND+60. This effect was, however, reversed later in adulthood at ND+90 via anti-serotonergic mechanisms. These data also provide a reference point for prospective studies investigating the effects of early-life psychotropic drugs on effects later in life. Furthermore it was suggested by the study that early-life treatment with venlafaxine did not significantly affect the anxiety or cognition of the stress sensitive rats when compared to their control, the FRL rats.

When taking all of the data and its suggestions into account, it seems that rodents (and potentially humans) that are genetically susceptible to have depressive-like behaviour could benefit from early-life treatment with venlafaxine. The beneficial effects in early adulthood may eventually be reversed and whether this is true in humans needs to be established. Humans are more complex, both neurobiologically

and psychologically, so that the outcome in humans may not be the same. Lastly, the small number of animals, the incomplete series of neurobiological markers and general weakness of the data from the current study needs to be considered, so that the findings may need confirmation in follow-up studies.

### **5.3 Recommendations for prospective studies**

Prospective studies should address both the deficiencies of the current study and explore promising avenues. This way it will give a more complete reference to the effects of early-life treatment with venlafaxine on the behaviour and neurobiological markers of antidepressant action in adulthood.

- Firstly the behavioural tests need to be repeated, firstly under improved, stabilized housing conditions and using a larger number of animals per treatment group, thereby providing sufficient statistical power to the data.
- The age of behavioural testing may be optimised and since the data suggest optimal effects at ND+60, studies may focus on measuring effects at this age.
- Treatment regimens may also be optimised and prospective studies may investigate administration of venlafaxine during pre-adolescence and adolescence.
- An expansion of the neurochemistry may also be of value and should include determining the concentrations of all the catecholamines and their metabolites, and not just *L*-NE and 5-HT. In particular, it may be useful to implement real-time measurements of monoamine levels using *in vivo* microdialysis. In addition, the neurochemistry should also be expanded to determine the levels of BDNF, SERT, NET as well as receptor density studies of NE- and 5-HT-receptors. The levels of BDNF will give an indication of the neuroplasticity and resistance to stress (§2.1.4.4) and it is necessary to determine whether the treatment regimen changed the BDNF levels in any way. The importance of the NE- and 5-HT-receptor densities can't be underestimated as the treatment is given during the development of these pathways and might influence the development (§2.1.3). If these receptor densities are influenced, it could have far reaching effects for the individual later in life.

#### **5.4 Limitations to the current study**

Limitations to the current study include that the study was conducted in rodents and not in humans. Even though the animal model of depression shows many similarities to depression in humans, it can't simulate all the intricacies of human depression. It is also important to note that the study is limited to only FSL rats.

In the current study there were a number of significant limitations that occurred. During the time that the study was conducted, the NWU Vivarium (animal centre) underwent a major upgrade. Animals had to be moved and housed at a different facility and they were returned just prior to commencement of the current study. Acclimatization of stress-sensitive rats at a new environment may take several months and generations. For example, we have seen repeatedly in the past that breeding returns to normal only after almost a year of acclimatisation. Even offspring may be affected by maternal stress, so that adaptation is possible only after several generations.

The work done in the facility was also not completed when the study commenced, so that there were several stressors (particularly noise) in the environment that was not ideal. This enhanced stress can be expected to have an effect on the behaviour of the rats, as well as the number of pups being born, and the outcome of the various treatments. These factors contributed to the low number of animals in the study. This study therefore needs to be repeated in a more controlled environment.

# Appendix A Congress Contribution

Appendix A contains the contributions made by myself at the 3's Company congress held in Cape Town from 4-6 October 2013.

## **Abstract**

### **The Effect of Early-Life Exposure of Rats to Venlafaxine on Behaviour and Neurobiological Markers of Antidepressant Action in Adulthood**

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## **Introduction**

Recent epidemiological studies have shown there is a persistent escalation in the diagnosis of major depressive disorder (MDD) in children and adolescents. This has been accompanied by an escalation in the prescription rate of antidepressants, specifically fluoxetine and escitalopram, for this age group. In fact, these two selective serotonin reuptake inhibitors are the only drugs currently approved by the Federal Drug Agency of the United States for the treatment of depression in children and adolescents. A black box warning has been issued about a potential increase in suicidal ideation during therapy onset (i.e. short-term untoward effect). This said little is known about the long-term neurodevelopmental effects of antidepressant treatment in children and adolescents, and particularly whether early-life treatment may affect the susceptibility to and development of psychiatric illness in adulthood. The objective of the current study was to investigate in stress-sensitive rats the effects of early-life chronic treatment (pre-natal and/or postnatal) with venlafaxine on behavioural and neurobiological biomarkers later in life. The pre-natal and postnatal phases described above are associated with marked development of both the serotonin and noradrenalin neurological pathways, justifying the use of the dual action serotonin-noradrenalin reuptake inhibitor venlafaxine. The aforementioned behavioural and neurological biomarkers represent measurements of cognition, anxiety-like and depressive-like behaviour and monoamine levels in the prefrontal cortex and the hippocampus.

## **Methods**

For the current study we used stress-sensitive Flinders Sensitive Line (FSL) male rats and their controls, Flinders Resistant Line (FRL) male rats, employing 12 rats per treatment group. For the treatment of prenatal animals, pregnant dams were injected subcutaneously with 10 mg/kg venlafaxine or saline for 14 days from natal day (ND)-15 to ND+02. For treatment of postnatal animals, new-born pups were injected subcutaneously with 3 mg/kg venlafaxine or saline for 14 days from ND+03 to ND+17. These doses were determined from previous studies in our laboratory. Following these drug treatments, all rat groups were subjected to a battery of behavioural tests, including the object recognition test (ORT), locomotor activity test (LOCO - Digiscan®), elevated plus maze (EPM) and forced-swim test (FST) on either ND+21, ND+35, ND+60 or ND+90 (with separate treatment groups for each age group). After the behavioural tests animals were decapitated, the brains removed and the prefrontal cortex and hippocampi were dissected. These were snap frozen and stored at -80°C until use, which entailed measurement of monoamine levels with high pressure liquid chromatography (HPLC) and electrochemical detection. All animal procedures were approved by the Ethics Committee of the North-West University (approval number: NWU-00045-10-S5), and are in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

## **Results**

As expected, saline-treated stress-sensitive FSL rats displayed significantly enhanced depressive-like behaviour in the relative to corresponding FRL rats, as observed at ND+35, ND+60 and ND+90. Importantly, depressive-like behaviour in FSL rats was reversed following prenatal, postnatal or both with venlafaxine relative to saline in FSL rats at ND+60, but not at ND+35 or 90, suggesting a delayed response appears in early adulthood but is overcome later in adulthood. Conversely, preliminary data from the ORT, LOCO or EPM did not reveal any significant differences between the various FSL treatment groups, including at ND+60.

## **Conclusions**

The implication of the current data therefore suggest that the early-life administration of venlafaxine to FSL rats (but not FRL rats) induce a delayed reversal of

depressive-like behaviour, manifesting at ND+60, but not at ND+35 or 90. Preliminary data do not support similar changes in anxiety-like behaviour or cognition.



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