# The effect of early-life exposure of stress-sensitive rats to the serotonin-norepinephrine reuptake inhibitor venlafaxine on behaviour in adulthood

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# Abstract

Major depressive disorder (MDD) is a serious psychiatric disorder with an increasing prevalence world-wide. It is estimated to affect 5% of the world's population, and has been estimated that by the year 2020 it will have become the second leading cause of disability in age groups 15 to 44 across both genders. MDD affects children and adolescents at an equally alarming rate, with the treatment options for these young patients mainly restricted to selective serotonin reuptake inhibitors (SSRIs) which have shown escalating prescription numbers of alarming proportion over the past few decades. Of further concern is that many human foetuses are exposed to antidepressants already in utero. Although available data have not suggested any major adverse effects in new-born babies following antidepressant-use by pregnant mothers, there is still a great deal of uncertainty with regard to any potential neurodevelopmental or other long-term effects that may manifest later in life.

The aim of the current study was to investigate, in stress-sensitive and control resistant rats, the long-term effects of early-life administration of the serotonin-norepinephrine reuptake inhibitor (SNRI) venlafaxine on cognition and behaviour later in life.

Pregnant dams of stress-sensitive Flinders sensitive line (FSL) rats, and their behavioural control Flinders resistant line (FRL) rats, received daily subcutaneous (s.c.) injections for fourteen days either saline (Sal) or 10 mg/kg venlafaxine (Ven), starting on prenatal day 15 (PreND-15). Similarly, new-born pups received daily s.c. injections for fourteen days either Sal or 3 mg/kg Ven, starting on postnatal day 3 (PostND03). In all cases there were four treatment groups respectively receiving prenatal plus postnatal injections as follows: Sal+Sal, Ven+Sal, Sal+Ven or Ven+Ven. For all treatment groups of both rat lines behavioural and cognitive tests were performed on PostND21, 35 and 60 and consisted of the forced swim test (FST), locomotor activity test (Digiscan®), novel object recognition test (nORT) and elevated plus maze (EPM). The behavioural tests measured the depressive-like behaviour (FST), locomotor activity (Digiscan®), memory consolidation (nORT) and anxiety-like behaviour (EPM) of the animals, following venlafaxine treatment during the different developmental stages in life.

A pilot study confirmed that roughly four male pups, on average, are delivered by a

pregnant dam, but with recorded variation of between 2 and 5.8 male pups per litter. The sex distribution per litter was also found to be approximately 50:50 for both FRL and FSL dams. This data were used to determine the number of dams to include per treatment group, yielding the required number of male pups.

Early-life venlafaxine treatment did not induce any significant changes in the depressive-like behaviour of FRL rats on PostND21, 35 or 60, or in that of FSL rats tested on PostND21 or 35. The treatment did, however, significantly decrease the depressive-like behaviour of the FSL rats on PostND60. This decrease was not accompanied by alterations in the overall locomotor activity. In fact, locomotor activity was altered only in FSL rats on PostND21 following pre- and postnatal venlafaxine treatment, suggesting a transient change during neurodevelopment. Cognition, as measured in the novel object recognition test, was reduced only in FRL rats at PostND60 following pre- and postnatal venlafaxine treatment. Since a similar change was not observed in FSL rats, the data suggest that the neurodevelopmental consequences of early-life antidepressant administration on cognition may be less harmful in stress-sensitive rats than in normal controls, i.e. pathology-dependent pharmacology. Finally, whereas the FSL rats displayed significantly decreased anxiety-like behaviour at PostND21, compared to FRL controls and not at any other age, it was concluded that this may also be a transient behaviour. The anxiety-like behaviour of both rat lines remained unaffected, following pre- and/or postnatal venlafaxine treatment, at any age.

In conclusion, early-life venlafaxine administration induced selective behavioural and cognitive effects in stress-sensitive rats, most likely due to effects on neurodevelopment. Whereas the most prominent effects manifested at PostND60, these effects may also be dependent on rat line, further suggesting a role for genetic predisposition in drug response.

# Opsomming

Major depressie is ’n ernstige psigiatriese afwyking wat wêreldwyd toeneem. Daar word beraam dat 5% van die wêreld se bevolking deur depressie geraak word en dat dit teen die jaar 2020 die naasbelangrikste oorsaak van ongeskiktheid vir persone tussen die ouderdomme van 15 en 44 jaar vir beide geslagte sal wees. Kinders en adolessente word ook in al hoe 'n groter mate deur major depressie geraak, met behandelingsopsies vir hierdie jong pasiënte hoofsaaklik beperk tot selektiewe seretonien heropname remmers (SSRI’s), waarvan die voorskrifsyfers oor die laaste dekade dramaties toegeneem het. Verdere kommer word gewek deur die feit dat menslike fetusse alreeds *in utero* blootgestel word aan hierdie antidepressante. Alhoewel beskikbare data nog geen ernstige newe-effekte aangetoon het in pasgebore babas wat tydens swangerskap blootgestel is aan antidepressante nie, is daar groot onsekerheid oor die moontlike effekte op neuro-ontwikkeling asook langtermyneffekte van hierdie geneesmiddels later in die jong pasiënt se lewe.

Die doelwit van die huidige studie was om die langtermyneffekte van ’n serotonien-norepinefrien heropname remmer, venlafaksien, op die kognitiewe- en gedragspatrone van stressensitiewe en -weerstandige rotte te ondersoek ná behandeling gedurende vroeë lewe.

Swanger wyfies van beide die stressensitiewe Flinders sensitiewe lyn (FSL)- rotte en hul gedragskontrole, Flinders weerstandige lyn (FWL)- rotte, het daagliks subkutaneuse inspuitings van ’n soutoplossing (Sout) of 10 mg/kg venlafaksien (Ven), vir veertien dae vanaf prenatale-dag-15 (PreND-15) ontvang. Pasgebore rotte is ook soortgelyk daagliks subkutaneus met Sout of 3 mg/kg Ven behandel vir veertien dae. In alle gevalle was daar vier behandelingsgroepe wat onderskeidelik prenataal en postnataal soos volg behandel is: Sout+Sout, Ven+Sout, Sout+Ven of Ven+Ven. Vir alle behandelingsgroepe is beide rotlyne gebruik vir die gedrags- en kognitiewe toetse wat op PostND21, -35 en -60 uitgevoer is. Hierdie toetse het die geforseerde swemtoets, ‘n lokomotoraktiwiteitstoets, nuwe voorwerp herkenningstoets en die verhoogde plus doolhof ingesluit. Die doel van hierdie gedragstoetse is om onderskeidelik die depressie-agtige gedrag, lokomotoraktiwiteit, geheue-konsolidasie en angstigheidsgedrag van die diere ná behandeling met venlafaksien gedurende die verskillende ontwikkelingsfases, te meet.

'n Loodsstudie het bevestig dat daar per pasgebore werpsel 'n gemiddeld van vier manlike rotte voorkom met 'n variasie van tussen 2 en 5.8. Daar is gevind dat die geslagsverspreiding per werpsel ongeveer 50:50 vir beide FSL- en FWL- wyfies sal wees. Hierdie data is gebruik om vas te stel hoeveel wyfies per behandelingsgroep ingesluit moet word om die nodige hoeveelheid manlike pasgebore rotte te verkry.

Venlafaksienbehandeling gedurende vroeë lewensontwikkeling het geen statisties-betekenisvolle veranderinge in die depressie-agtige gedrag van FWL-rotte, gemeet op PostND21, -35 en -60, geïnduseer nie. Hierdie behandeling het wel 'n statisties-betekenisvolle verlaging in die depressie-agtige gedrag van FSL-rotte op PostND60 tot gevolg gehad. Geen verandering in lokomotoraktiwiteit het met hierdie verlaging in depressie-agtige gedrag in die diere gepaard gegaan nie, in teendeel, 'n verandering in lokomotoraktiwiteit kon slegs waargeneem word by FSL-rotte wat pre- en postnatale behandeling met venlafaksien ontvang het – 'n aanduiding van 'n verbygaande verandering in die neuro-ontwikkeling. Kognisie, soos gemeet in die nuwe voorwerp herkenningstoets, was slegs statisties-betekenisvol verlaag in die pre- en postnataal venlafaksien-behandelde FRL-rotte op PostND60. Aangesien daar nie 'n soortgelyke verandering in die FSL-rotte opgemerk is nie, kan daar uit die data afgelei word dat die neuro-ontwikkelingsgevolge van vroeë-lewe antidepressantblootstelling op kognisie minder skadelik in stressensitiewe rotte as in normale kontroles is. Laastens het die FSL-rotte, in vergelyking met FRL-kontroles, aansienlik minder angstige gedrag geopenbaar op PostND21 as op enige ander ouderdom, wat daarop dui dat hierdie tipe gedrag ook 'n verbygaande verskynsel in hierdie rotlyn mag wees. Die angstigheidsgedrag van beide rotlyne was onveranderd na pre- en/of postnatale venlafaksienbehandeling, op alle ouderdomme waarop die diere getoets is.

Ten slotte, venlafaksienbehandeling gedurende vroeë lewe induseer selektiewe gedrags- en kognitiewe veranderinge, heel waarskynlik as gevolg van effekte op neuro-ontwikkelling. Die mees prominente effekte is eers teen PostND60 waargeneem en mag ook afhanklik wees van die rotlyn, wat die rol van genetiese vatbaarheid in geneesmiddelbehandeling verder versterk.

"NOT BY MIGHT

NOR BY POWER,

BUT BY MY SPIRIT,

SAYS THE

LORD ALMIGHTY"

---

Zech. 4:6

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“*The process of maturing doesn’t mean to become a captive of conceptualization. It is to come to the realization of what lies in our innermost selves*.” – Bruce Lee

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# Declaration

I, Stephanus Frederik Steyn hereby declare that all experimental work, planning, literature research, data capturing and interpretation, and writing of this dissertation was conducted by myself, under the guidance of my supervisor (Prof CB Brink) and co-supervisor (Prof BH Harvey).

_____ _____

**SF Steyn** **Date**

*(student)*

As supervisors, Prof CB Brink and Prof BH Harvey, we confirm that the above statement by Mr SF Steyn is true and correct.

_____ _____

**Prof CB Brink** **Date**

*(supervisor)*

_____ _____

**Prof BH Harvey** **Date**

*(co-supervisor)*

# Introduction

(All abbreviations are listed in *Addendum E*)

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

## 1.1 Dissertation approach and layout

The dissertation is presented in an article format, where the key data is presented as an article (*Chapter 3*) for publication in an accredited journal. All supplementary data is presented in an addendum (*Addendum B*). In addition the literature review and conclusions concerning the study is taken up into separate chapters in this dissertation. The following outline serves to assist the reader where to find key elements of the study inside the dissertation:

- **Problem statement, study objectives and study layout:**
  Chapter 1 (*Introduction*)
- **Literature background:**
  Chapter 2 (*Literature review*) and

  Chapter 3 (*Article introduction*)
- **Materials and methods:**
  Chapter 3 (*Article methods*) and

  Addendum A (*Materials and methods*)
- **Results and discussion:**
  Chapter 3 (*Article*) and Addendum B (*Additional results*)
- **Summary and conclusions:**
- Chapter 4 (for the study as a whole, including the article and addendum).

## 1.2 Problem statement

It is estimated that more than 120 million people worldwide suffer from depression and that this psychiatric disorder will become the second leading cause of disability by the year 2020 (World Health Organization, 2011a). This estimate, however, does not discern differences in the incidence within defined age groups, such as in children, adolescents, adults and geriatrics. It is clear from numerous studies that this disorder affects people irrespective of race or socio-economic status, while there appears to be an increase in susceptibility as a function of age, not only for depression, but for other psychiatric conditions as well (Harrington *et al*., 1990; Lewinsohn *et al*., 2000).

Nevertheless, children and adolescents are not exempt from this disorder. Until the 1970's there was debate on whether depression could in fact affect children (Malkesman and Weller, 2009), while sobering epidemiological studies thereafter clearly suggested that they are indeed affected (Birmaher *et al*., 1996; Costello *et al*., 2006; Keenen *et al*., 2004; Kessler *et al*., 2001) and that the number of children diagnosed and treated for major depression has increased dramatically, not only due to better diagnosis, but also due to an actual increase in the incidence of anxiety-related disorders amongst children and adolescents in developing countries (Zito and Safer, 2001; Zito *et al*., 2002).

The genetic aspect of major depression is very limited with regards to data, but several studies have shown that there is indeed a familial factor in the development of major depressive disorder (MDD) and suggests that the onset of MDD in patients under the age of thirty years is mostly familial (Weissman *et al*., 1984). Further data has indicated that children who come from a family with a history of MDD that goes back two generations are at a higher risk for developing the same condition (Weissman *et al*., 2005).

Currently, the research data on the use of antidepressants in the treatment of depression in children and adolescents are limited, particularly due to potential long-term neuropsychiatric effects. In the United States of America (USA) the Food and Drug Administration (FDA) has approved fluoxetine (Prozac®), a selective serotonin reuptake inhibitor (SSRI), as the drug of choice for treatment of depression in

children and adolescents (Bylund and Reed, 2007; Wagner, 2005), with a warning that it may cause a dangerous initial increase in suicidal thoughts and ideation (Wagner, 2005).

Several sets of data suggest that SSRIs, such as fluoxetine, are clinically more effective in the treatment of major depression in children and adolescence than are tricyclic antidepressants (TCAs) (Bridge *et al*., 2007; Hazell *et al*., 1995; Kratochvil *et al*., 2006; Mason *et al*., 2009; Ryan, 2003; Whittington *et al*., 2004). The best plausible explanation for this phenomenon (different from results in adults) is that serotonergic neurodevelopment starts and matures earlier (maturation before the onset of adolescence) than noradrenergic neurodevelopment (maturity in early adulthood). Importantly, SSRIs target the serotonergic pathways exclusively (or preferentially) whereas TCAs target both the serotonergic and noradrenergic pathways and preferentially the noradrenergic pathways (Choi *et al*., 2009; 2010; Findling and McNamara, 2004; Lewis, 1998; Murrin *et al*., 2007).

Since the number of SSRI prescriptions for children and adolescents has been on the increase (Zito and Safer, 2001; Zito *et al*., 2002), it has also become of utmost importance to understand the long-term effects thereof on neurodevelopment and the relapse in the development of psychiatric disorders later in life. Currently the benefit of using antidepressants to treat severe MDD (to counteract the serious symptomatology and risk of suicide) is to outweigh the risk of acute side-effects. Pre-clinical studies in rats, however, suggest that exposure to psychotropic drugs early in life induce neurochemical changes in the developing brain, of which the effects can only be observed later in life (Choi *et al*., 2009; 2010; Noorlander *et al*., 2008).

Taking the above-mentioned, neurochemical changes into account, it needs to be considered that early-life treatment of humans with antidepressants may indeed affect neurodevelopment, as well as behaviour and cognition later in life.

Our laboratory therefore aims to determine the long-term neurobiological, neurobehavioural and cognitive effects of early-life exposure to psychotropic drugs in stress-sensitive rats, compared to a stress-resilient control line. Within this umbrella project, the current study is a pilot study that aims to establish an appropriate early-life (prenatal and early childhood) exposure regimen to psychotropic drugs, as well as to determine an appropriate age later in life i.e. postnatal day 21, 35 and 60

(PostND21, 35 and 60) to measure any behavioural and cognitive changes. For this project we have used the serotonin-norepinephrine reuptake inhibitor (SNRI) venlafaxine, since this drug will reveal more concerning any effects resulting from enhancement of both serotonergic and noradrenergic pathways.

## 1.3 Study objectives

The primary objective of the current study was to determine whether pre- and/or postnatal administration of the SNRI antidepressant, venlafaxine, has any effects on anxiety- and depressive-like behaviour and/or cognition (see below) later in life in stress sensitive Flinders sensitive line (FSL) rats.

Secondary objectives included will be to:

- Validate an appropriate pre- and postnatal treatment regimen with venlafaxine versus vehicle control;
- Determine the appropriate age later in life (i.e. PostND21, 35 or 60) to observe any of the behavioural and cognitive changes listed below and
- Determine whether stress sensitive (FSL) rats respond differently to treatment than their control line, the Flinders resistant line (FRL) rat.

The specific ages of PostND21, 35 and 60 were chosen to replicate specific stages in the developmental process of the animal. PostND21 represents an early childhood stage at which animals have been successfully tested in previous studies (Bylund and Reed, 2007), PostND35 represents the adolescent stage, as sexual maturity occurs during the fifth week after birth (Murrin *et al*., 2007; Zeinoaldini, 2005), while PostND60 represents an early stage in adulthood.

## 1.4 Study layout

In the current study venlafaxine or vehicle-control was administrated to pregnant FSL and FRL rat dams from prenatal day 15 (PreND-15) to PreND-01 as well as to the young pups of these dams from PostND03 to PostND14.

Each treatment group consisted of 8 rats (4 rats x 2 trials/condition). All animals received the following treatments subcutaneously (s.c.) during the pre- and postnatal stages of life (indicated as treatment prenatal and postnatal treatment respectively):

- saline and saline (vehicle control) (*coded* ***Sal+Sal***);
- venlafaxine and saline (*coded* ***Ven+Sal***);
- saline and venlafaxine (*coded* ***Sal+Ven***) and
- venlafaxine and venlafaxine (*coded* ***Ven+Ven***).

Thereafter the rats were housed as normal until PostND21, 35 or 60, when anxiety-like and depressive-like behaviour as well as cognition was determined by a battery of behavioural tests:

- Cognitive function *(Novel object recognition test)*
- Locomotor activity *(Digiscan® animal activity monitor)*
- Anxiety-like behaviour *(Elevated plus maze)*
- Depressive-like behaviour *(Forced swim test)*

Venlafaxine was chosen for this pilot study, since it inhibits the reuptake of both serotonin and norepinephrine, and will thus reveal any neurobehavioural and cognitive changes via either of the two mechanisms. In fact, the systematic review by Bylund and Reed suggest that these pathways affect neurodevelopment differently (Bylund and Reed, 2007), while venlafaxine would then target both neurotransmitters and therefore screen for all changes induced by either or both of these signalling cascades.

Regarding the above, venlafaxine will result in an overview of the possible changes that may develop later in life, when administered to young rats. At the end of the study, the behavioural tests focussing on norepinephrine and serotonin directed behaviours will show whether or not any norepinephrine and serotonin-mediated changes are observed in these animals. From these results, future studies using selective noradrenergic and serotonergic drugs can define whether it is the influence on the serotonin and/or noradrenergic pathways that result in these specific behavioural changes.

The doses and dosing schedules of venlafaxine for the treatment of pregnant dams and pups were selected based on previous studies. Pregnant dams received 10 mg/kg s.c. of venlafaxine (Folkessen *et al*., 2010; Larsen *et al*., 2010; Scaini *et al*., 2010), whereas the pups received 3 mg/kg (s.c.) (Dawson *et al*., 1999).

## 1.5 Ethical approval

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.

# Literature review

Major depressive disorder (MDD) is a serious mood disorder and this chapter will discuss the neuropsychological and neurobiological aspects, as well as the pharmacological treatment thereof, with the focus point being MDD in children and adolescents. The first section will cover the definition, diagnostic criteria, signs and symptoms and epidemiology of MDD, focusing on its manifestation in children, adolescents and pregnant women. Focussing on the juvenile brain, the second section will discuss relevant neurobiology (including the anatomy, neurophysiology and neurodevelopment) of the human and rodent brains. The third section will reflect on the current understanding of the neuropathology of MDD, particularly in children and adolescents. Finally, the different neurobiological hypotheses of major depression, as well the various drug treatment strategies available for major depression will be discussed along with the current hypotheses concerning long-term effects of the drugs.

## 2.1 Major depressive disorder

Depression is a neuropsychological condition that generally manifests as sadness and feelings of hopelessness and/or worthlessness. However, most individuals will experience such feelings as a normal response to stressful life events at some point during their life. 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). MDD is considered a serious psychiatric disorder, particularly due to its disabling nature and the increased suicidal risk associated with severe forms of the disorder (Bylund and Reed, 2007; Fava and Kendler, 2000).

MDD is one of the oldest known medical conditions, dating back to ancient Greece (Fava and Kendler, 2000) but was first recognized as a biochemical phenomenon in the mid nineteen sixties. In the modern world, MDD has been estimated to be the most costly brain disorder in Europe with a total cost of the disorder corresponding to 1% of the total annual European economy (Sobocki *et al*., 2006). In a European

study during 2004, the total cost of depression was estimated to be around 118 billion Euros, of which nine billion Euros was spent on drug cost alone (Sobocki *et al*., 2006). The amount generated from selective serotonin reuptake inhibitor (SSRI) sales in the USA exceeded ten billion Dollars in a single year (Nestler *et al*., 2002).

Depression can affect people of all ages, race and economical classes and influences virtually all aspects of existence, including the individual's psychological, social, mental and even biological wellbeing, resulting in alterations in both personal and professional spheres of life.

The World Health Organization (WHO) estimated that MDD affects approximately 121 million people worldwide and is the fourth most important cause of loss in disability-adjusted life years worldwide (Kiss, 2008; Longone *et al*., 2008; Rex *et al*., 2004). Furthermore it is estimated that MDD will also become the second leading cause of disability by the year 2020 in the age group 15-44 years of both genders combined (World Health Organization, 2011a).

Of concern is that only about one third of patients with MDD achieve total remission in response to a single antidepressant (Trivedi *et al*., 2006), whereas about one third 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 patients of such a young age has been investigated in a number of studies. However, the long-term effects of such treatments on neurodevelopment and psychological outcome still remain unclear.

### 2.1.1 Epidemiology

It has been estimated that 2-5% of the global population suffers from MDD (Bylund and Reed, 2007). There is, however, demographic variation throughout the world and in the United States of America (USA) MDD is estimated to affect between 4.1% and 10% of the population annually (Kessler *et al*., 1994a; Waraich *et al*., 2004), while data suggests a 9.7% lifetime prevalence in the Republic of South Africa (RSA) for a major depressive episode (MDE) which is higher than the data of prevalence for any mood disorder, reported by the WHO in Nigeria (3.3%). This is however

significantly lower than the lifetime prevalence of the United States of America (21.4%) for any mood disorder (Tomlinson *et al*., 2009). It has also been established in several studies that patients living with HIV-AIDS are particularly prone to develop depression (Hays *et al*., 1992; Janssen *et al*., 1989; Judd and Mijch, 1996; Ostrow *et al*., 1989; Perdices *et al*., 1992). Therefore, in light of the high prevalence of HIV-AIDS in Africa and in particular in RSA, this has strong local relevance.

Research suggests that the incidence of MDD in women is almost twice of that in men (Earls, 1987; World Health Organization, 2011b) and according to the National Comorbidity Study of the USA the lifetime prevalence of MDD in women has been estimated to vary between 17% and 21.3%, compared to 12.7% observed in men (Blazer *et al*., 1994; Ververs *et al*., 2006).

The data discussed above suggest a significant prevalence of MDD in adults, there are also several studies demonstrating an alarming increase in the prevalence of MDD in children and adolescents (discussed in *§ 2.1.1.1)*.

### 2.1.1.1 Major depressive disorder in children and adolescents

As mentioned above, depression can affect anyone of any age and even though depression in children and adolescents were ignored until the early 1970's (Malkesman and Weller, 2009), it is now a known fact that this condition is present in these young patients and that the number of incidents are on the increase.

It has been estimated that 25% of children will have experienced a MDE by the time he or she reaches adulthood (Kessler *et al*., 2001). More specifically, 2.8% of children under the age of thirteen years and 5.6% of adolescents older than fourteen, but younger than eighteen, have been diagnosed with MDD (Costello *et al*., 2006), where less than 1% of these were aged younger than eight years (Keenen *et al*., 2004). It is thus clear that MDD has a very strong childhood and/or adolescent onset and has been documented by a number of studies (Angold *et al*., 1998; Christie *et al*., 1989; Kessler *et al*., 1994b; Merikangas *et al*., 1994; Lahey *et al*., 1996; Lewinsohn *et al*., 1994).

Data suggest a strong heritability factor for MDD. For example, an individual with a first-degree relative (i.e. father or mother) suffering from MDD, has a risk of up to 42% for developing the same condition (Sullivan *et al*., 2000). In addition, children belonging to a family with two or more generations affected by MDD are at significantly higher risk (60%) of developing MDD or related psychiatric disorders later in life (Weissman *et al*., 2005). Taking this, as well as the seriousness of MDD into account, it is therefore not surprising that pharmacotherapeutic intervention in children presenting with MDD is an important and a justified consideration. But as the research on the long-term safety and efficacy on the available antidepressants for these young patients are very limited, one has to weigh the known immediate effects of the condition against the possible, unknown long-term effects of the treatment in order to help these young patients. The available data on the safety and efficiency of antidepressant in children is discussed in *§ 2.2* and *§ 2.3.2.*

In fact, the use of antidepressants in children represents one of the fastest growing treatments in the psychiatric community (Zito and Safer, 2001; Zito *et al*., 2002). Prescription rates for fluoxetine rose 1.8-fold between 1991 and 1995 in elementary and preschool children in the USA (Zito *et al*., 2000), whereas a different study indicated as much as a 10-fold increase in the use of SSRIs in children five years of age and younger, between 1993 and 1997 in Canada (Minde, 1998). The reason for this significant difference between the USA and Canada is not known, but may possibly be the result of different prescribing protocols. In a study done by Delate and colleagues (Delate *et al*., 2004) on the same age group of children (i.e. five years and younger), a 0.64-fold increase in prescription rates for boys and a 1-fold increase in girls of the same age were indicated in the USA between 1998 and 2002.

Depression in children and adolescents has been associated particularly with memory impairments (Günther *et al*., 2004), low self-esteem (Renouf *et al*., 1997; Stavrakaki *et al*., 1991) and an increased risk for suicidal behaviours (Fava and Kendler, 2000; Weissman *et al*., 1999) and substance abuse (Lubman *et al*., 2007). These consequently interfere with the academic and social development and functioning, including functioning within support systems such as families (Wagner, 2005).

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

As mentioned above, children are likely to develop MDD, but it is not the only means by which they are exposed to antidepressant drugs. Pregnant women also subject foetuses to these drugs via crossing of the placenta or excretion in the breast milk to the new-born babies (Kinney *et al*., 2007). It is thus important to discuss the prevalence and other relevant data on pregnant or lactating women.

Women are at their highest risk for developing depression during the childbearing years (Blazer *et al*., 1994), which can be as high as 9 to 16% (Bennett *et al*., 2004; Evans *et al*., 2001; Josefsson *et al*., 2001; Oberlander *et al*., 2006). This, along with the fact that women are twice more likely to develop MDD than men (Earls, 1987; World Health Organization, 2011b), contributes to the risk that pregnant woman may take antidepressants, either by continuing therapy initiated prior to pregnancy, or by starting therapy during pregnancy (Gentile and Galbally, 2011; Field, 2010; Nonacs *et al*., 2005; Ververs *et al*., 2006).

Data suggest that 0.5% of women will start antidepressant treatment during pregnancy (Ververs *et al*., 2006) and as much as 25% of depressed women, already on antidepressant therapy, will continue therapy during pregnancy, as discontinuation of antidepressant therapy during pregnancy significantly increases the rate of relapse of depression in the mother (Cohen *et al*., 2006). The drug of choice in this specific group of patients is fluoxetine (Nonacs and Cohen, 2003), which, along with other SSRIs, have shown a dramatic increase in prescription rates in pregnant woman over the last two decades (Andrade *et al*., 2008; Cooper *et al*., 2007; Oberlander *et al*., 2006; Vaswani *et al*., 2003; Ververs *et al*., 2006). That the benefit-risk ratio is considered favourable in many instances should however not create a false sense of safety with the use of these drugs during pregnancy. There is no, or limited, evidence that neurodevelopment is not altered or that major foetal malformations is not a potential risk (Louik *et al*., 2007), particularly in predisposing individuals.

So what are the potential benefits for the foetus (without considering here the needs of pregnant mother) for the use of antidepressants during pregnancy? Depression during pregnancy is associated with an increased risk of preterm delivery, low birth

weight and admission of the new-born to the neonatal intensive care unit (Bonari *et al*., 2004; Chung *et al*., 2001; Field *et al*., 2010). Furthermore, other adverse effects affecting neurodevelopment such as developmental delay (Deave *et al*., 2008), lowered IQ in adolescence (Hay *et al*., 2008) and impaired language development (Nulman *et al*., 2002; Paulson *et al*., 2009) have been associated with maternal and/or perinatal depression. These consequences are believed to be prevented by effective treatment of the maternal depression.

It is clear that the child's dependency on the mother (including her well-being) is thus of concern when considering the advantages of the treatment for both mother and child and that mother-to-child exposure of antidepressants plays a vital part of the study objectives as discussed in *§ 1.3.*

## 2.1.2 Diagnosis

According to the Diagnostic and Statistical Manual of Mental Disorders 4$^{th}$ ed. (DSM-IV) an episode of MDD, in an adult patient, is diagnosed when one of the first two symptoms, plus any other four, listed below (*Table 2-1, page 13*), presents for at least two weeks and causes a disruption in the normal daily functioning of the individual (American Psychiatric Association, 1994). Furthermore the American Academy of Family Physicians (AAFP) recommends that the criteria for the diagnoses of MDD in children and/or adolescents are not different from those used for adult patients (American Academy of Family Physicians, 2000).

**Table 2-1: Diagnostic criteria for the diagnosis of MDD according to the DSM-IV.**

- Depressed mood, but can present as irritable mood in children and adolescents;
- Markedly diminished interest or pleasure in activities;
- Significant weight loss or gain when not dieting;
- Insomnia or hypersomnia;
- Psychomotor agitation or retardation;
- Fatigue or loss of energy levels;
- Feelings of worthlessness or inappropriate guilt;
- Diminished ability to think or concentrate;
- Recurrent thoughts of death and suicide.

From the criteria used to diagnose MDD it is clear that the diagnosis of this condition is not based on objective diagnostic tests, but rely on a set of variable and relatively subjective symptoms. Thus, major depression may be viewed as a heterogeneous syndrome, presenting with varying patterns of a number of distinctive symptoms (Liebenberg *et al*., 2009).

## 2.1.3 Signs and symptoms

Overall, the clinical presentation and course of MDD are believed to be the same across childhood, adolescence and adulthood (Kovacs, 1996).

MDD in children, adolescents and adults causes 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. In addition there is also an increase in risk for self-harm, suicide ideation and thoughts of death. Depression in childhood may also promote the development of a personality disorder in susceptible individuals, since the depression interferes with the developing personality (Andersen and Navalta, 2004; Bylund and Reed, 2007; MERCK 2006; Weissman *et al*., 1999).

## 2.1.4 Neurobiology (anatomy and neuropathology)

Several brain areas have been associated with the development, manifestation and prognosis of MDD. Areas identified to play a key role include the prefrontal cortex (PFC), hippocampus and amygdala, as depicted in *Figure 2-1*. The neuropathophysiology of depression has been studied most extensively in adults, so that much of our basic understanding thereof results from data on the adult brain. However, a limited number of neurobiological studies have suggested that the brain regions affected in depression in childhood are comparable to that affected in adults (Andersen and Navalta, 2004; Kowatch *et al*., 1999). Importantly, there is also evidence of important neurophysiological and neuroanatomical differences in these affected areas, discussed below.

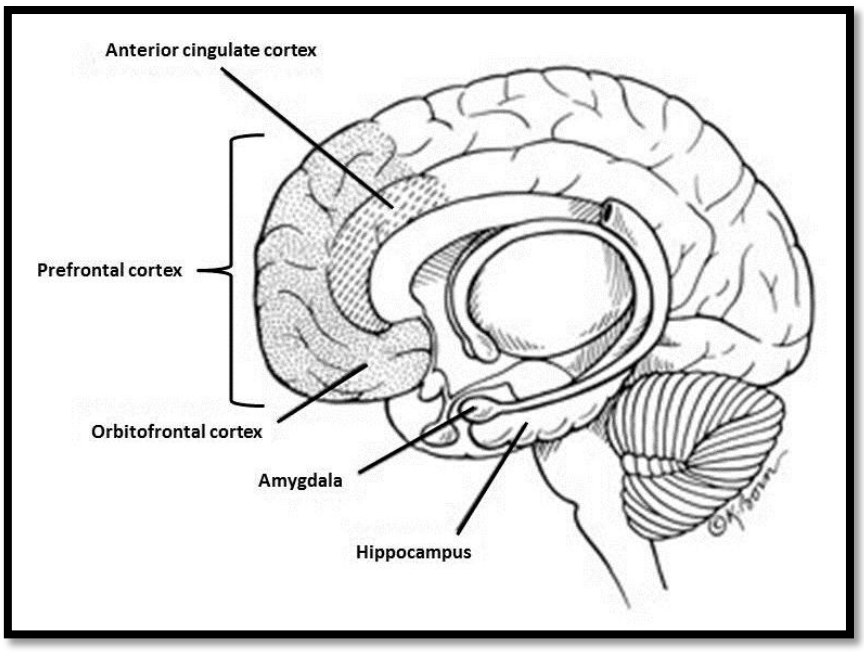


**Figure 2-1: Illustration of the three major areas affected by MDD (DANA, 2011).**

### 2.1.4.1 The prefrontal cortex

Key cognitive processes are mediated by the prefrontal cortex (PFC). The impact of these processes has been elucidated by the following eloquent description: "*The medial prefrontal cortex (MPFC) is associated with meta-cognitive representations that enable us to reflect on the values linked to outcomes and actions (that is,*

*thinking about thinking). These high level representations have a major role in many aspects of social cognition. Not only do they allow us to reflect in the values that other people attach to actions and outcomes, they also allow us to reflect on what other people think about us*" (Amodio and Frith, 2006).

During adolescence, the PFC (*Figure 2-1, page 14*) undergoes the most fundamental and prolonged changes, as compared to the changes seen in regions such as the primary motor and sensory cortices (Bourgeois *et al*., 1994; Huttenlocher, 1979). The first of these changes involve pruning of the synapses, along with a reduction in grey matter. The second is the increase in myelination, which is correlated with an increase in white matter (Giedd *et al*., 1999).

In children suffering from depression, it has been demonstrated that a number of changes do occur in the PFC, when compared to the normal development in non-depressed controls. Depression evokes changes such as reduced frontal white matter, increased frontal grey matter (Steingard *et al*., 2002) and larger left-sided, but not right-sided prefrontal cortical volumes (Steingard *et al*., 2000). Furthermore reduced regional cerebral blood flow (rCBF) in the left anterofrontal lobe of the brain has been demonstrated (Tutus *et al*., 1998), as well as dysfunction of the frontal lobe as measured by electrophysiological readings (Steingard *et al*., 2000).

The above-mentioned changes that occur in children or adolescents suffering from MDD are consistent with that observed in adults (Andersen and Navalta, 2004; Kowatch *et al*., 1999).

### 2.1.4.2 The hippocampus

The hippocampus (*Figure 2-1, page 14*) plays a key role in learning and verbal memory (Reiman, 1997).

Several studies have suggested that a smaller left hippocampal size is present in patients suffering from depression when compared to healthy controls (Bremmer *et al*., 2000; Frodl *et al*., 2003; MacMaster and Kusumakar, 2004; MacQueen *et al*., 2003), while earlier studies failed to demonstrate volume changes (Ashtari *et al*., 1999; Bookheimer *et al*., 2000; Vakili *et al*., 2000).

More recent data, using better technology, measuring techniques such as MRI and voxel-based morphometry and experimental design, have now demonstrated more clearly a reduction in the size of the left hippocampus in severely depressive patients with a long history of the disorder. In fact, the hippocampal volume decreased with as much as 4-5%, compared to healthy controls (Campbell *et al*., 2004; Frodl *et al*., 2008; Videbech and Ravnkilde, 2004). What has not been clearly demonstrated is to which extent this change reflects altered neuronal structure, neuronal body volume, synaptic sprouting, and total water, protein and lipid content.

Early studies have shown that stress, as associated with MDD, does influence hippocampal neurogenesis and plasticity (Reagan and McEwen, 1997; Woolley *et al*., 1990). The increased, chronic stress (resulting in chronic elevation of cortisol levels, discussed in *§ 2.1.6.3*) has been associated with dendritic remodelling of the synaptic terminal structures (Sapolsky, *et al*., 1985; 1990; Sousa *et al*., 2000; Uno *et al*., 1989), resulting in cell death in certain brain regions (Czéh and Lucassen, 2007; Harlan *et al*., 2006; Sousa and Almeida, 2002).

Juvenile patients with a familial history of MDD has been demonstrated to present with a decreased hippocampal volume, suggesting that even at such an early age these individuals may be at a higher risk for developing MDD later in life (MacMaster *et al*., 2008). This finding is supported by data indicating that a decrease in hippocampal volume was present in adult male patients suffering from a first-time MDE (Frodl *et* al., 2002). Gender thus seems to be an important factor in the development of depression and the differences between male and female patients need to be further investigated.

In summary, there is currently general consensus that MDD is associated with impaired hippocampal function and reduced volume, whereas the exact nature and clinical implications of such changes are less well understood. Consequently a large number of studies continue to investigate these phenomena in search of interventions that may prevent or effectively treat MDD.

### 2.1.4.3 The amygdala

Located deep within the anterior inferior temporal lobes (*Figure 2-1, page 14*) the amygdala plays an important role in processes such as fear and experiences of negative effects, as well as of the perception of emotional stimuli (Aggleton, 1993) to direct emotional responses and psycho-social behaviours (Baxter and Murray, 2002; Drevets, 2003).

Changes in the size of the amygdala have been observed in patients with affective disorders (Altshuler *et al*., 1998, 2000; Mervaala *et al*., 2000; Sheline *et al*., 1998; Strakowski *et al*., 1999; Tebartz van Elst *et al*., 2000), for example an increase in amygdala volume in patients suffering from temporal lobe epilepsy with co-morbid depression has been documented (Tebartz van Elst *et al*., 2000). Whereas no current data support a significant difference in the amygdala volume of patients suffering from recurrent episodes of depression compared to healthy control patients, patients with a first MDE have been shown to present with an increased amygdala volume when compared to patients with recurrent MDEs (Frodl *et al*., 2003; Mervaala *et al*., 2000; Sheline *et al*., 1998).

Volume sizes of the amygdala core, which include the amygdala basal nucleus, lateral nucleus and accessory basal nucleus, were found to be smaller in depressed female patients compared to their male counterparts (Sheline *et al*., 1998). However, a smaller volume in right amygdala was found to be similar across both sexes (Mervaala *et al*., 2000).

According to several studies, the amygdala is a highly plastic brain structure in which new cells are continually generated into adulthood (Carrillo *et al*., 2007; Keilhoff *et al*., 2006). 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 is believed to result in an increased risk for the development of psychiatric disorders.

In summary, the several anatomical changes have been reported throughout the brain, in children and adolescents suffering from MDD, as discussed above. In addition to these changes, the changes in especially the PFC and amygdala differed

between genders, suggesting that the development of the condition may differ between male and female, but needs further investigation.

### 2.1.5 Neurotransmitter pathway development

The rates of brain development differ markedly between species, whereas the general age-related pattern of neuronal maturation, using several neurobiological parameters, remains similar across most mammalian species. Various mammalian species have been studied in this regard, but with the most data available for rodents (and more specifically the rat) this available data on brain development will be discussed in more detail below and used as a baseline for further references.

There are several factors to take into account when comparing the development of the human brain to similar development in the rat brain. Firstly, it has been demonstrated that at birth the relative weight of the rat brain is comparable to that of the human brain by the second trimester. Secondly, rats reach sexual maturity at about five weeks of age, which corresponds to adolescence in humans (Murrin *et al*., 2007; Zeinoaldini, 2005). Since hormonal changes markedly affect brain development and since adolescence is also an important marker for certain hallmarks in brain neurobiological development, these comparative ages between human and rodent is also of importance when interpreting data on neurodevelopment.

A time-line demonstrating the relationship between age and serotonergic or adrenergic development, respectively, is depicted in *Figure 2-2* (*page 19*). Murrin and colleagues (Murrin *et al*., 2007) investigated changes in the serotonin and norepinephrine content of specific brain regions of the rat embryo during pregnancy. Their data demonstrated the presence of serotonin-containing neurons in the 8 mm rat embryo, whereas the norepinephrine-containing neurons were only first observed in the 11 mm rat embryo.

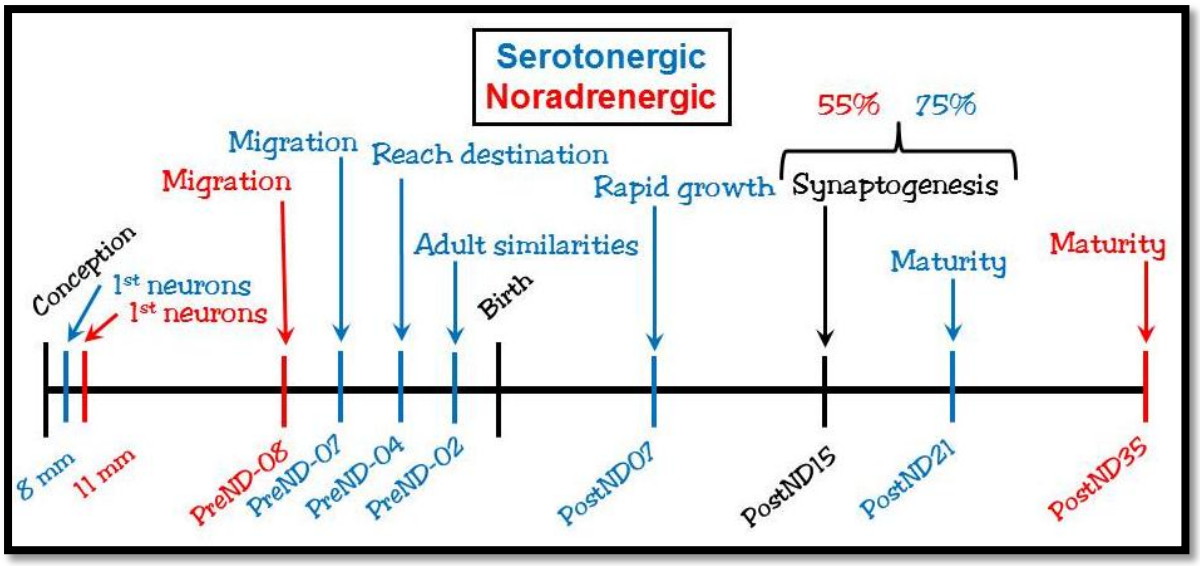


**Figure 2-2: Timeline illustration of the serotonergic and noradrenergic pathway development.**

The serotonergic neurons start projections to adult-like pathways by PreND-07 and reach their destination by PreND-04 (Wallace and Lauder, 1983). Two days before birth, the serotonergic pathways already show strong adult similarities with no significant change in the noradrenergic system apparent (Aitken and Tork, 1988; Wallace and Lauder, 1983).

Seven days after birth (i.e. PostND07), the serotonergic neurons show a rapid surge to levels exceeding levels seen in adult rats (Murrin *et al*., 2007). This rapid surge correlates with the increase in serotonin-labelled varicosities that increase by 20% compared to numbers present at birth (Dinopoulos *et al*., 1997). After the significant increase, the levels start to decrease to normal adult levels around postnatal week three (Andersen and Navalta, 2004).

The pattern of development of adrenergic neurons is somewhat different from that of serotonergic neurons. Here migration of cortical adrenergic neurons is initiated at about day thirteen of gestation (i.e. PreND-08) and continues throughout early postnatal development.

By PostND15 synaptogenesis of the serotonin pathway is at approximately 75% of adult levels in the raphe nucleus of the rat brain, whereas the norepinephrine synaptogenesis is at only 55% of adult levels in the locus coeruleus part of the brain at the same time (Lauder and Bloom, 1975). Serotonin 5-$HT_7$ receptor types are expressed by PostND15, but are virtually absent by PostND21 (Vizuete *et al*., 1997)

and may be a result of “synaptic pruning”. Maturation of cortical neurons, i.e., sprouting of cellular processes and formation of synaptic contacts with neighbouring neurons, occurs mainly within the first three weeks of postnatal development, which is the time when noradrenergic interventions is increasing to adult levels (Berger-Sweeney and Hohmann, 1997; Markus and Petit, 1987).

By PostND21, the serotonergic system reaches maturity, while the noradrenergic system continues to develop throughout postnatal development and only reach maturity by PostND35 or the fifth postnatal week (Murrin *et al*., 2007).

From the data above it is clear that the most fundamental development of serotonin pathways occur mainly during the prenatal developmental phase, with final maturation in the first few postnatal weeks. The noradrenergic system, in contrast, mainly develops during postnatal development with final maturation numerous weeks after the serotonin system (Murrin *et al*., 2007).

Comparing the noradrenergic development information, discussed above, with the human brain, a similar timeline is followed in the development of this neurotransmitter. The norepinephrine neurotransmitter is already detectable at around five to six weeks of gestation. The levels of norepinephrine, furthermore, correlates with that of the rat, as it increases throughout the first trimester, especially from two months of gestation where after a decrease of 30-40% occurs between six months and early childhood (Murrin *et al*., 2007).

Documented data indicates that it is not only in the rat that the serotonin system reaches maturity earlier than the noradrenergic system, but in other species as well (as shown in *Table 2-2, page 21*).

**Table 2-2: indicators of maturation of adrenergic and serotonergic systems in the mammalian brain (Murrin *et al*., 2007).**

| Parameter/Species | Reaches adult levels | |
|---|---|---|
| | **Norepinephrine** | **Serotonin** |
| **Innervation/Rat** | 5 weeks | 3 weeks |
| **Innervation/Monkey** | 2 years | 2 weeks |
| **Neurotransmitter/Rat** | 5 weeks | 3 weeks |
| **Neurotransmitter/Monkey** | 2 years | 2 months |
| **Neurotransmitter/Cat** | >11 weeks | 3 weeks |
| **Transporters/Rat** | 3 weeks | Birth |

As mentioned, it has been hypothesised that the same timeline (*Table 2-2*) for neurotransmitter development is followed in several species, including human beings. This hypothesis has been the reasoning for the increased clinical effectiveness of the selective serotonin reuptake inhibitors (SSRIs) versus the tricyclic antidepressants (TCAs) (Bridge *et al*., 2007; Hazell *et al*., 1995; Kratochvil *et al*., 2006; Mason *et al*., 2009; Ryan, 2003; Whittington *et al*., 2004).

As will be discussed in *§ 2.1.7.3*, the SSRIs preferably target the serotonin system, which develops earlier than the noradrenergic system, which is mainly targeted by the TCAs (see *§ 2.1.7.2*). Targeting these developing systems have been associated with so-called “miswiring” of the specific pathways, and possibly resulting in increased risks for other serotonin-related behaviours and disorders such as anxiety (Bagdy, 1998; Graeff, 2002), depression (Berendsen, 1995; Nutt *et al*., 1999) and schizophrenia (Kusljic *et al*., 2003). Possible hypotheses for these enduring effects, caused by antidepressants (and other psychotropic drugs), which may only be observed later in life is discussed in *§ 2.4*, while the different hypotheses of MDD is discussed in *§ 2.1.6*, below.

## 2.1.6 Hypotheses of major depressive disorder

There is a general consensus that neurobiological changes underlie depression, and that the aetiology thereof may be best described by a model that accounts for both environmental and biological causes. Drug treatments address primarily the

neurobiological basis, and hence several hypotheses about the neurobiological basis of depression have emerged. In fact, the drugs that are effective in treating depression have prompted the recognition of a biological basis and the realization that a better understanding thereof is essential in the discovery of novel drug targets for the treatment of depression. In addition, a few attempts have been made to find a unifying hypothesis to explain all observations and to incorporate all other hypotheses. Hence, this section will discuss the various hypotheses and the evidence to support them as well as reflect on their strengths and weaknesses.

### 2.1.6.1 The monoamine hypothesis

Since the mid 1960's, the monoamine hypothesis became the most widely studied and accepted hypothesis to describe to neurobiological basis of depression (Schildkraut, 1965). This hypothesis, as originally formulated and also its subsequent modifications, states that depression is caused by a deficit in monoaminergic neurotransmission, involving norepinephrine, serotonin and/or dopamine at certain key sites in the brain (Katzung, 2007; Schildkraut, 1965).

The monoaminergic hypothesis originated from the observation that the anti-hypertensive drug reserpine, a potent monoamine neurotransmitter stores depleting drug, resulted in depressive-like symptoms in many patients (Katzung, 2007; Sapolsky, 2000; Schildkraut, 1995). These depressive-like symptoms were reversed by drugs that increase the monoamine levels, such as monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants (TCAs) (Schildkraut, 1965). Today, however, we know that the selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) also increase the monoamine, serotonin, which leads to a decrease in depressive-like symptoms.

The mechanisms by which the levels of monoamines can be elevated include:

- Blocking the reuptake of monoamines from the synapse;
- Inhibiting the intraneuronal metabolism of the monoamine or
- Blocking the pre-synaptic inhibitory auto- and hetero-receptors.

The monoamine hypothesis is not a flawless theory and a number of shortcomings have been identified since its inception:

- Firstly, certain drugs, for example cocaine and amphetamines, increase brain monoaminergic activity, but are not clinically effective as antidepressants;
- secondly, not all depressed patients respond equally to the same antidepressant and
- finally, and most importantly, an increase in monoamine levels at a synaptic level is detectable only hours after of the administration of antidepressants, whereas antidepressant effects are seen only after continuous administration of these drugs for a number of weeks (Baldessarini, 1989).

During the past two decades the monoamine hypothesis has been modified in an attempt to more accurately describe depression. The modified theory suggests that the acute increase in monoamine levels at a synaptic level may only be an early step in a potentially complex cascade of events which ultimately results in antidepressant activity (Piñeyro and Blier, 1999). Furthermore, the prolonged onset time of therapeutic effect has been attributed to the desensitization of inhibitory auto- and hetero-receptors following increased synaptic monoamine levels. The blockade of nerve terminal auto-receptors have been demonstrated to enhance the therapeutic response to antidepressants, also supporting the notion that antidepressant effects result from long-term adaptive changes in the monoamine auto- and hetero-regulatory receptors (Elhwuegi, 2004).

The monoamine hypothesis has played an important role in the development of new antidepressants, in particular the development of the SSRIs, TCAs and SNRIs.

### 2.1.6.2 The cholinergic super-sensitivity hypothesis

The cholinergic super-sensitivity hypothesis was first introduced in the early 1970's by Janowsky and colleagues (Janowsky *et al*., 1972) and postulates that depression and mania are associated with hyper- and hypo-cholinergic states, respectively, which correspondingly bolsters accompanying 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. The organophosphates inhibit acetylcholinesterase (AChE), resulting in increased acetylcholine (ACh) levels throughout the brain and periphery (Gershon and Shaw, 1961). Administration of a centrally acting AChE inhibitor, such as physostigmine, but not a peripherally active agent, neostigmine, has been shown to evoke depressive-like symptoms in human patients (Janowsky *et al*., 1974).

Furthermore, the Flinders sensitive line (FSL) rats, a validated animal model of depression, have an increased number and function of high-affinity nicotinic acetylcholine receptors (nAChRs) (Auta *et al*., 2000; Tizabi *et al*., 2000) resulting in a predisposed cholinergic sensitivity (*§ A.1.1*) and ultimately strengthens the cholinergic super-sensitivity hypothesis as has been confirmed in a recent clinical study (Perlis *et al*., 2002). Further support of the hypothesis, is that the anti-muscarinic drug, scopolamine, has been shown to display antidepressant-like effects in rats (Brink *et al*., 2008; Furey and Drevets, 2006; Janowsky, 2007).

However, the cholinergic-super-sensitivity hypothesis of depression has limitations as all anticholinergic drugs are not effective antidepressants. There seems to be a fine balance between the activation and desensitization of the cholinergic receptors for pro- or antidepressant effects to manifest and it is mostly viewed a contributor, but not necessarily a critical determinant of mood (Picciotto *et al*., 2008).

Finally, the anticholinergic activity of antidepressant drugs, such as the TCAs, is associated with bothersome side-effects and increased discontinuation rates which resulted in the development of additional hypotheses for the development of MDD.

### 2.1.6.3 The hypothalamic-pituitary-adrenal-axis hypothesis

The third hypothesis for the development and pathophysiology of MDD is the hypothalamic-pituitary-adrenal-axis (HPA-axis) hypothesis, which states that chronic increased levels of corticosteroids lead to decreased normal biological neurogenesis and manifests as MDD (Hoschl and Hajek, 2001; Mizoguchi *et al*., 2003; Sheline *et al*., 1996).

The HPA-axis plays an important role in the body's response to acute and/or chronic stress. Corticotrophin-releasing hormone (CRH) is released in the paraventricular nucleus (PVN) of the hypothalamus, which in turn stimulates the anterior pituitary to secrete adrenocorticotrophin (ACTH). The ACTH goes on to stimulate the adrenal glands which results in the release of glucocorticoids (cortisol in humans and corticosterone in rodents) which have an extensive effect on the immune system, behaviour, general metabolism as well as certain brain functions (Ehlert *et al*., 2001; Herbert *et al*., 2006; Schimmer and Parker, 2001). This hormonal pathway is normally suppressed by the negative feedback inhibition pathway of cortisol on the hypothalamus as illustrated in *Figure 2-3*.

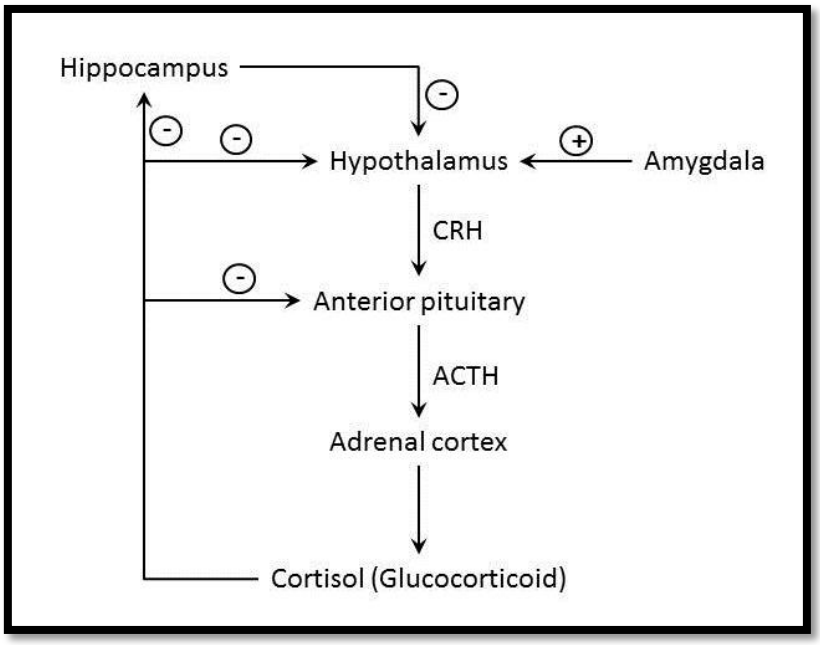


**Figure 2-3: Illustration of a normal functioning HPA-axis in the human brain.**

It was more than fifty years ago that Board and colleagues (Board *et al*., 1957) reported elevated cortisol levels in depressed patients where after Carroll and colleagues reported a failure to suppress endogenous cortisol secretion in depressed patients after being administered dexamethasone (Carrol *et al*., 1981). This inability to suppress cortisol secretion indicates a dysfunction of the negative feedback system of the HPA-axis (*Figure 2-3*) and is the cornerstone of the HPA-axis hyperactivity hypothesis.

Further studies have reported increased cortisol levels in the saliva, urine and plasma of depressed patients (Nemerhoff and Vale, 2005) as well as in adult female patients, who were either physically or sexually abused during childhood and who were now depressed as adults (Heim and Nemerhoff, 2002).

Studies suggested that non-steroidal anti-inflammatory drugs with central action such as celecoxib and reboxetine have been associated with antidepressant activities as adjunctive therapy for MDD patients (Akhondzadeh *et al*., 2009; Müller *et al.,* 2006) further supporting the HPA-axis hypothesis of MDD.

Finally, data have indicated that cortisol levels return to normal once depression has remitted (Caetano *et al*., 2004; Hoschl and Hajek, 2001) and is possibly a solid indicator that the HPA-axis hyperactivity hypothesis has relevance.

### 2.1.6.4 The neuroplasticity hypothesis

According to the Oxford dictionary, plasticity refers to the adaptability of an organism to change in its environment or differences between its various habitats (Oxford Dictionaries, 2011). This would implicate that neuroplasticity would refer to the ability of neurons to adapt to the changing needs of the brain, either in terms of structure or function.

As discussed in *§ 2.1.4* several changes have been associated with brain regions in patients suffering from long-term MDD. These anatomical changes, along with chronic stress, associated with MDD, have led to the neuroplasticity hypothesis. This hypothesis implies that a decrease in neurotrophic factors, which regulate plasticity in the brain, are present in patients suffering from long-term MDD (Aydemir *et al*., 2006; Duman and Monteggia, 2006; Karege *et al*., 2002; 2005; Manji *et al*., 2003; Shimizu *et al*., 2003).

Two of the major neurotrophic factors are the transcription factor cyclic adenosine monophosphate response element binding protein (CREB) and the brain-derived neurotrophic factor (BDNF). These factors (along with others) were first characterized for regulating neuronal growth and differentiation during development, but are now known to be potent regulators of plasticity and survival of adult neurons

(Ghosh *et al*., 1994; Mamounas *et al*., 2000; Sklairtavron and Nestler, 1995). The decrease in neurotrophin expression, especially BDNF, has been associated with chronic stress, as experienced by patients suffering from MDD (Duman and Monteggia, 2006).

The resulting anatomical changes associated with decreased levels of neurotrophic factors are apparent on cellular and molecular levels. The cellular changes include reduced neurogenesis and cell death, while the molecular changes include modified gene expression, protein synthesis and phosphorylation (Reay *et al*., 2010).

Data has indicated that several classes of antidepressants, such as the MAOIs, SSRIs, and the SNRIs have been associated with increased BDNF levels supporting the relevance of the neuroplasticity hypothesis, as these drugs result in a decrease of depressive-like symptoms (Duman and Monteggia, 2006; Nibuya *et al*., 1995).

### 2.1.7 Treatment options for major depressive disorder

Five main classes of antidepressants can be distinguished. The classification is usually done on the basis of neurobiological mechanism of action and hence these classes are expected to differ regarding clinical efficacy and adverse effect profile. In fact, the search and discovery of different classes of antidepressants has been driven by the search for drugs that would be effective in treatment-resistant cases, display an earlier onset of action and exhibit greater tolerability. Each of the different classes of antidepressants currently available is discussed below, with reference to their mechanisms of action illustrated in *Figure 2-4* (*page 28*).

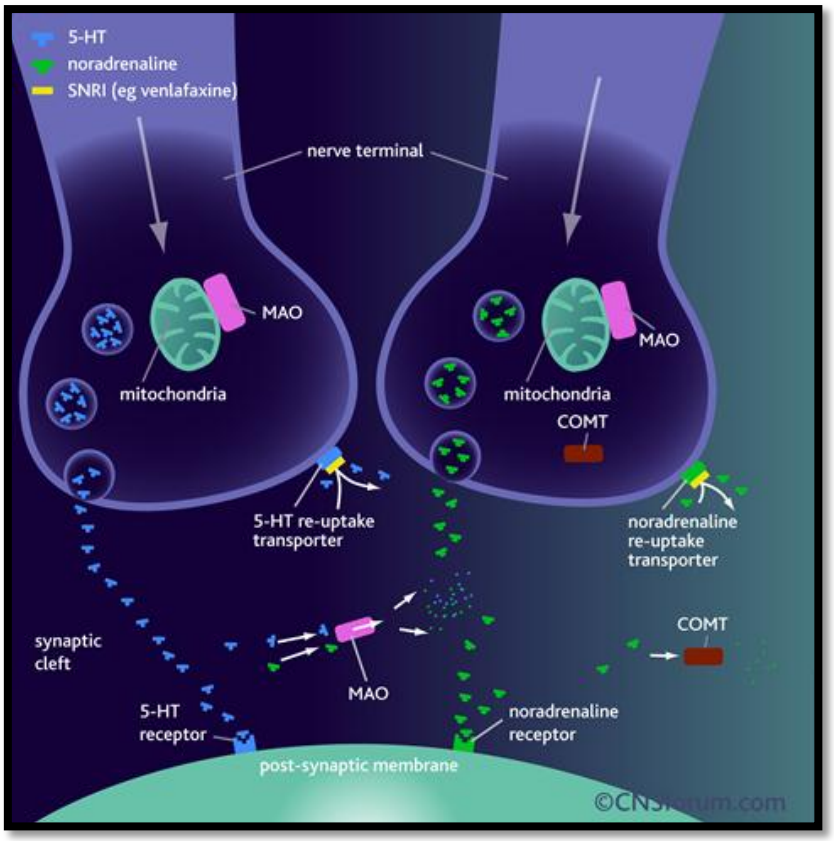


**Figure 2-4: Illustration of neurotransmitter action in the synaptic cleft (CNSForum, 2011).**

*Figure 2-4* illustrates the central synapse with its monoaminergic receptors and neurotransmitters. Each of the five classes of antidepressants affects these neurotransmitters and receptors in a different way and is discussed in more detail below.

### 2.1.7.1 The monoamine oxidase inhibitors

Monoamine oxidase (MAO) is an enzyme present in the outer mitochondrial membrane of neuronal and non-neuronal cells (*Figure 2-4*). Two isoforms of MAO are present in the human body, namely MAO-A and MAO-B. These enzymes are responsible for degrading the monoamines *l*-norepinephrine (*l*-NE), serotonin (5-HT) and dopamine (DA) in the synaptic cleft, resulting in a decrease in their concentrations which may, according to the monoamine hypothesis (*§ 2.1.6.1*), lead to the manifestation of depression (Finberg and Gillman, 2011; Ruhe *et al*., 2007; Schildkraut, 1965).

The MAOIs act by inhibiting this specific enzyme in the pre-synaptic neurons resulting in increased levels of available monoamines in the synaptic cleft (Katzung, 2007; Schildkraut, 1995) which is believed to improve depressive-like symptoms.

Tranylcypromine and phenylzine are examples of two of the older MAOIs which non-selectively inhibit both MAO-A and MAO-B. These drugs induce a hypertensive crisis when taken with dietary tyramine, a monoamine precursor, because of the acute increase in monoamines throughout the body, without the ability to degenerate these toxic levels of monoamines. This hypertensive crises is known as the "cheese effect" (Finberg and Gillman, 2011).

MAO-A primarily catabolises *l*-NE and 5-HT, leading to MAO-A selective inhibitors, such as moclobemide and brofaromine, being primarily used as antidepressants (Lotufo-Neto *et al*., 1999). The first MAO-B inhibitor, selegeline (Knoll and Magyar, 1972; Knoll *et al*., 1978), however, is used in the treatment of Parkinsonism since MAO-B preferentially metabolises DA (Brunton *et al*., 2010a). However, this drug has shown promise as an antidepressant in patients with co-morbid depression at higher dosages (Katzung, 2007).

In summary, the MAOIs, mainly decrease depressive-like symptoms by enhancing monoaminergic neurotransmission which correlates with the monoamine hypothesis.

### 2.1.7.2 The tricyclic antidepressants

The tricyclic antidepressants (TCAs) have a characteristic three-ring nucleus and have been used as antidepressants for over three decades (Katzung, 2007; Reay *et al*., 2010). These drugs are chemically similar to phenothiazine drugs and thus have similarities in side-effect profile i.e. affinities for the $H_1$-, α-adrenergic- and muscarinic receptors (Leonard, 1997). The antidepressant activity of the TCAs was discovered during clinical trials in schizophrenic patients, who showed limited improvement in psychotic behaviour, but increased beneficial effects on their depressive symptoms following TCA treatment (Hollister, 1981).

The mechanism of action of these drugs is primarily, but not exclusively, the inhibition of the reuptake of *l*-NE, leading to increased *l*-NE levels in the synaptic

cleft. Apart from inhibiting the norepinephrine transporter (NET) (*Figure 2-4*), the TCAs also block $H_1$-, 5-$HT_2$-, $\alpha_1$- and muscarinic receptors (Brunton *et al*., 2010b), resulting in an increased adverse profile (Wijeratne and Sachdev, 2008).

Two subclasses of TCAs are notable, namely secondary and tertiary amines, which differ with regard to their spectrum of selectivity for reuptake transporters and activity on various specific receptors. These differences include the tertiary amines inhibiting both *l*-NE and 5-HT and the secondary-containing amines preferably inhibiting *l*-NE (Brunton *et al*., 2010c).

The tertiary amines, such as amitriptyline, clomipramine and imipramine, are metabolized in the liver to secondary amines (desipramine, nortryptiline and maprotiline), which are pharmacologically active and are responsible for both the therapeutic and toxic effects. In addition, the secondary amines are metabolized to pharmacologically inactive compounds (Reay *et al*., 2010).

In summary, the TCAs became the mainstay of antidepressant therapy, but with an increased adverse effect profile, led to the development of the SSRIs (*§ 2.1.7.3*), but are still used, alternatively to SSRIs, in the treatment of MDD today.

### 2.1.7.3 The selective serotonin reuptake inhibitors

In the late 1960's Nobel Prize winner Carlsson and colleagues, proposed brain 5-HT as an alternative target for antidepressant treatment (Carlsson *et al*., 1968; Lapin and Oxenkrug, 1969). This led to the discovery of numerous SSRIs in the early 1980s, notably fluoxetine. Eventually, almost two decades after its discovery, fluoxetine entered the market as antidepressant under the trade name Prozac® manufactured by the pharmaceutical company Eli Lilly® (Stokes and Holtz, 1997). Many SSRIs followed, including paroxetine, citalopram and sertraline (Furgeson, 2001; Kasper *et al*., 2009), all commonly used as antidepressants today (Fuller, 1995).

The SSRIs are believed to treat depression by inhibiting the reuptake of 5-HT, caused by SERT (serotonin transporter) into the pre-synaptic terminal and consequently increasing the 5-HT concentration in the synaptic cleft (*Figure 2-4,*

*page 28*). This synaptic 5-HT increase, spurs numerous secondary effects via the stimulation and desensitisation of pre- and post-synaptic 5-HT receptor subtypes, and eventually the modulation of serotonergic neurotransmission resulting in antidepressant effects.

Stimulation of the 5-$HT_{1A}$-autoreceptor (Artigas *et al*., 1996) and the 5-$HT_{1B}$- receptor has been described as the critical target for the anti-depressive-like effects of the SSRIs (Blier, 2003). These receptors suppress 5-HT synthesis, but repeated stimulation leads to gradual down regulation and desensitization and ultimately to an increase in the synthesis and release of 5-HT (Blier *et al*., 1987; 1990; Chaput *et al*., 1991). Stimulation of the 5-$HT_3$-receptor subtype is associated with adverse effects of these specific antidepressants, such as gastrointestinal and sexual effects. The agitation or restlessness, which is also associated with SSRI treatment, may be linked to the stimulation of the 5-$HT_{2C}$-receptor subtype (Baldessarini, 2001).

The SSRIs have been associated with less adverse effects, yet comparable clinical efficacy than their progenitors, and has therefore become the mainstay of antidepressant therapy today. In addition, this class of antidepressants are the only FDA approved treatment for children and adolescents suffering from MDD (Bylund and Reed, 2007; Wagner, 2005) as well as pregnant women (Mulder *et al*., 2011). However, a delayed onset of action, frequent treatment resistance and a number of troublesome side-effects, yet less troublesome than the TCAs, remains a problem (Hindmarch, 2001).

### 2.1.7.4 The serotonin-norepinephrine reuptake inhibitors

Based on the inter-individual variation of the aetiology and symptoms of depression, the high frequency of treatment resistance, variation of clinical efficacy of antidepressants with an array of mechanisms of action and our better understanding of the neurobiological basis of depression (see *§ 2.1.6*), resulted in MDD being viewed as a heterogeneous disorder. Drugs belonging to the SNRIs include venlafaxine, duloxetine and milnacipran.

Venlafaxine was first introduced in the early 1990's by the Wyeth® pharmaceutical company and will be discussed in more detail, as it was the drug used in the current study.

The monoamine hypothesis (*§ 2.1.6.1*) is still the most extensively studied hypothesis and in this regard both 5-HT and NE has been strongly associated with MDD (Bylund and Reed, 2007; Montgomery, 1997). The SNRIs were designed to target both of these neurotransmitter pathways as many of the older TCAs have been proven to block both 5-HT and NE reuptake (seen in *Figure 2-4, page 28*), but only at higher dosages, leading to decreased depressive symptoms (de Oliveira *et al*., 2004; Entsuah *et al*., 2001; Gur *et al*., 1999; MERCK 2006), but with a marginal selectivity for serotonin reuptake.

In addition, venlafaxine is a moderate inhibitor of the reuptake of the neurotransmitter dopamine (DA) (Ellingrod and Perry, 1994), yet with a significantly lower potency than for 5-HT reuptake (i.e. a 13- to 130-fold selectivity for 5-HT relative to DA) (Bolden-Watson and Richelson, 1993; Muth *et al*., 1986). Selectivity for *l*-NE relative is between 3- and 17-fold lower relative to that of 5-HT (Béïque *et al*., 1998; Bolden-Watson and Richelson, 1993; Muth *et al*., 1986; Owens *et al*., 1997). This selectivity has neurophysiological relevance, as suggested by a study demonstrating that venlafaxine produces an inhibition of the dorsal raphe firing superior to that found with the SSRIs (Gartside *et al*., 1997). However, venlafaxine is a less potent inhibitor of *l*-NE reuptake than the TCAs (Bolden-Watson and Richelson, 1993).

Importantly, venlafaxine does not exhibit significant affinity for muscarinic-cholinergic-, $H_1$-, $D_2$- and/or benzodiazepine receptors and does not inhibit monoamine oxidase (Cusack *et al*., 1994; Muth *et al*., 1986; Owens *et al*., 1997). Accordingly, it is associated with less adverse effects than the TCAs or the older MAOIs (Roseboom and Kalin, 2000), and is also better tolerated.

Some data suggest that the SNRIs, and in particular venlafaxine, may have an earlier onset of action compared to other classes of antidepressants (Feigner, 1994; Montgomery, 1995). Several studies have found an initial clinical response to venlafaxine already at fourteen days after commencement of treatment, compared to

twenty one days with the SSRIs (Benkert *et al*., 1996, 1997; Clerc *et al*., 1995; Guelfi *et al*., 1995; Rudolph *et al*., 1991).

In addition to its antidepressant effects, venlafaxine exhibits analgesic effects, and hence has shown promise for the treatment of neuropathic pain (Enggaard *et al*., 2001) while other drugs in this class are used for urinary incontinence (i.e. duloxetine) (Brunton *et al*., 2010d).

### 2.1.7.5 The atypical antidepressants

The atypical antidepressants refer to drugs with unrelated chemical structures and mechanisms of action (drug targets) that differ from those discussed above. The development of the drugs in this class resulted from the need for drugs with a different spectrum of superior efficacy, with enhanced tolerability (i.e. fewer troublesome side effects) and an earlier onset of action (Kent, 2000). Although these drugs may in some instances be useful in patients that do not respond to other drugs, mostly due to inter-individual differences, they all affect monoaminergic neurotransmission in some way or another and have not been demonstrated to be superior in general.

The atypical antidepressants include the 5-HT receptor antagonists (trazodone, nefazodone and mirtazapine), melatonergic receptor agonist (agomelatine), NE-reuptake inhibitors (reboxetine and maprotiline), 5-HT reuptake enhancers (tianeptine) and the multiple action bupropion.

Trazodone, nefazodone and mirtazapine all preferably antagonize the 5-HT receptors, but affect other receptors as well (i.e. adrenergic and H-receptors) (Brunton *et al*., 2010e; Eison *et al*., 1990).

Agomelatine is claimed to have a novel mechanism of action, being an agonist on melatonergic receptors attempting to normalise disturbed sleep patterns in depressed patients as well as being an antagonist to the 5-$HT_{2C}$ receptors (San and Arranz, 2008).

The NE-reuptake inhibitors, such as reboxetine and maprotiline, exhibit antidepressant effects via the inhibition of the NET, resulting in increased *l*-NE levels in the synaptic cleft (*Figure 2-4, page 28*) (Katona *et al*., 1999; Wong *et al*., 2000).

Tianeptine, a tetracyclic antidepressant, presents with the same onset of action as the common antidepressants, but with a more favourable adverse effect profile (Wagstaff *et al*., 2001). The mechanism of action of this drug is unique in that it increases (and not inhibits) the reuptake of 5-HT (Fattaccini *et al*., 1990; Oritz *et al*., 1993), while recent studies have demonstrated that tianeptine buffers excitatory amino acid receptors against stress (Kole *et al*., 2002) in order to protect the brain morphology from adverse effects associated with stress (McEwen and Magarinos, 2001). Tianeptine does, however, affect the reuptake of *l*-NE or MAO activity (Brink *et al*., 2006). Pre-clinical data strongly suggest that tianeptine possesses neuroprotective activity in the hippocampus (Magariños *et al*., 1999; Czéh *et al*., 2001), an area in the brain closely associated with the neuropathology of depression.

Finally, bupropion acts via multiple mechanisms as it increases both noradrenergic and dopaminergic neurotransmission via inhibiting the reuptake systems as well as increasing presynaptic NE and DA release (Foley *et al*., 2006).

In summary, the atypical class of antidepressants reaches across a wide variety of mechanisms of action, all resulting in decreased depressive symptoms and increased tolerability for the patients.

## 2.2 Treating major depressive disorder in children and adolescents

The diagnosis criteria and symptoms of MDD in childhood and/or adolescents (discussed in *§ 2.1.2*) correlates with MDD in adults, but different treatments exist for the different age groups. When treating adult patients, a wide variety of antidepressants (discussed in *§ 2.1.7*), along with non-pharmacological treatment options such as electroconvulsive therapy (ECT) are available (Wijeratne and Sachdev, 2008).

Conversely, the treatment of MDD in children and adolescents has a limited amount of options. In June 2003, Britain's Medicines and Healthcare products Regulatory Authority (MHRA) banned six SSRIs, with the exception of fluoxetine, in the treatment of MDD in patients younger than eighteen years. This was done according to clinical data which indicated a 3-fold risk increase in self-harm and suicide in these young patients, compared to a placebo-controlled group (Andersen and Navalta, 2004; MHRA, 2003). In the USA, fluoxetine is currently the only FDA approved antidepressant for children and adolescents (Bylund and Reed, 2007; Wagner, 2005) and it is recommended that an SSRI should be used as monotherapy as first stage treatment and switched to an alternative SSRI if the first proved to be unsuccessful (Hughes *et al*., 1999).

As discussed in *§ 2.1.5*, the clinical efficiency of the SSRIs are hypothesized to be the result of the faster maturing serotonergic system, compared to the noradrenergic system in young patients. The data on the safety and efficacy of antidepressants targeting the noradrenergic system, such as venlafaxine, is very limited (Andersen and Navalta, 2004). Available data have not shown any significant safety and efficacy in juvenile patients treated for MDD. In a study done by Emslie and colleagues, no significant difference in depressive-like behaviour of children treated with venlafaxine and a placebo control group were evident. The study did, however, indicate a significant improvement in adolescents treated with venlafaxine (Emslie *et al*., 2004a). Furthermore, following six months of treatment with different doses of the extended release (ER) venlafaxine, no adverse effects were reported and a decrease in depressive symptoms were observed in patients aged between seven and seventeen (Emslie *et al*., 2004b).

Data on TCA treatment in adolescents and children, suggests no superiority to the placebo treated groups in a number of studies (Keller *et al*., 2001; Kutcher *et al*., 1994; Kye *et al*., 1996).

Regarding the treatment duration of MDD in children and adolescents, there is a correlation with the adult condition as a twelve month treatment period is recommended in these young patients (Pine, 2002). This treatment period is supported by data that indicated an 82% response rate for children treated for twelve

weeks, compared to a 75% response rate following six weeks of treatment (Emslie *et al*., 2004c).

In summary, the SSRIs are currently the preferred drug of choice in the treatment of MDD in children and adolescents and should be administered for prolonged periods in order to improve symptoms, while antidepressants targeting *l*-NE, needs to be further investigated as it may provide additional treatment options.

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

A number of pre-clinical and clinical studies relevant to the current study have been performed. The majority of these investigated the effects of SSRIs on foetal and neonatal neurodevelopment and on postnatal behaviour. However, the number of these studies performed with an SNRI, such as venlafaxine as implemented in the current study, is limited.

### 2.3.1 Pre-clinical studies

Recent evidence suggests that daily injections of fluoxetine to pregnant mice increased the anxiety- and depressive-like behaviour of the new-born pups in adulthood (Noorlander *et al*., 2008). When administrating pups from PostND04 to PostND21 with either fluoxetine or citalopram (Ansorge *et al*., 2008; Popa *et al*., 2008), similar effects on behaviour were reported.

Furthermore, citalopram administration from PostND08 to PostND21 in male rat pups, reduced sexual activity (Maciag *et al*., 2006), increased rapid eye movement (REM) sleep and decreased the latency to sleep onset (Popa *et al*., 2008) in adulthood. Slight body weight increases, reduced sexual behaviour and increased anxiety-like behaviour was observed in the animals that started paroxetine treatment on PostND21 (de Jong *et al*., 2006).

In another recent study with an atypical antipsychotic, risperidone, an increase in the 5-$HT_{1A}$-receptor number in the medial prefrontal cortex and hippocampus of juvenile animals were observed as well as a reduction in 5-$HT_{2A}$-receptor subtypes at a relative low dose of risperidone (Choi *et al*., 2010), suggesting that chronic treatment with a central acting psychotic, during early-life development, may alter the normal neurodevelopment which, in turn, may lead to effects only observed later in life.

### 2.3.2 Clinical studies

Since fluoxetine is the drug of choice during pregnancy (Nonacs and Cohen, 2003) and since it is the only antidepressant approved for use in children and adolescents (Bylund and Reed, 2007; Wagner, 2005), clinical studies relevant to the current project mainly report on the effects of SSRIs.

A recent study suggested that foetuses exposed to standard or higher than standard SSRI doses developed differently than unexposed controls, irrespective of SSRI type. These differences included increased motor activity of the foetuses 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). This observation may be explained by serotonergic neuron-mediated increase in motor neuron output, eventually facilitating repetitive movements of the foetus (Brancherau *et al*., 2002; Lucki, 1998).

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

## 2.4 Theoretical framework for enduring effects of drug action

As mentioned in *§ 1.3*, the main objective of the current project was to identify whether early-life administration of the antidepressant venlafaxine induces any long-term behavioural or cognitive effects in rodents evident later in life. In addition, the

study aimed to determine at which age later in life these effects are most robust. In this regard, several neurodevelopmental hypotheses have been proposed to explain and predict such effects.

The first hypothesis is the process of overproduction and pruning of synapses in the developing brain, as mentioned in *§ 2.1.5*. Several studies have suggested that the monoamine systems of mammals generally exhibit an overproduction in neurons, synapses and/or receptors during development, compared to levels in adult animals. After a period of time, this increased number of neurons, synapses and/or receptors decline to normal levels found in adult animals (Andersen *et al*., 2000; Andersen *et al*., 1997; Whitaker-Azmitia, 1991).

This process of decline is referred to as pruning and can be as much as 40% in the number of synapses lost during adolescence (Huttenlocher, 1979; Andersen *et al*., 2000). The overproduction and pruning process may create a period of vulnerability in which the different brain regions are more prone to long-lasting effects, caused by drugs affecting these transmission pathways, especially in brain areas where the process of overproduction and pruning is high compared to areas which have already reached adult levels (Andersen, 2003; Lidow and Song, 2001).

Two additional hypotheses have been suggested to the overproduction and pruning hypothesis. The first of which, known as the “neural Darwinism” hypothesis, was introduced by Edelman in the early nineties (Edelman, 1993). This hypothesis suggests that the brain “selects” the synapses that are to be retained into adulthood, which will ultimately allow it to match the needs of the environment (Piatelli-Palmarini, 1989; Teicher, 2002).

Secondly, the “instructionist” hypothesis proposes that the brain is “instructed” by the environment to develop in a certain manner, based on the structural and/or functional requirements of certain brain systems (Quartz and Sejnowski, 1997).

The brain’s synaptic development (i.e. sprouting, growth and formation) during early-life development is influenced by changing levels of DA (Gelbard *et al*., 1990; Kalsbeek *et al*., 1988; Lankford *et al*., 1988; Todd, 1992), NE (Feeney and Westerberg, 1990; Kline *et al*., 1994) and 5-HT (Lauder and Krebs, 1978; Kupperman and Kasamatsu, 1984; Whitaker-Azmitia and Azmitia, 1986). Juvenile

exposure to drugs affecting these neurotransmitter levels may cause the effects to only manifest later in life, when childhood or adolescence has reached its peak (Andersen and Navalta, 2004).

# Article

The current dissertation is presented in the “article format”, as recognised by the North-West University. Accordingly, the current chapter presents the core data as a research article in a chosen, appropriate scientific journal. Accordingly, an article titled:

*“The effect of early-life administration of venlafaxine in Flinders Sensitive Line (FSL) rats on cognition and depression and anxiety-related behaviour later in life.”*

was prepared as a full-length research report to be submitted to ***European Journal of Pharmacology***. Different from other chapters in this dissertation, the current chapter was prepared according to the Instructions to the author for this journal, as described in *Addendum C*. The references for this chapter are therefore also provided at the end of the manuscript, and not at the end of the dissertation as for other chapters.

# 3.1 Title page

***Title of article***

The effect of early-life administration of venlafaxine in Flinders Sensitive Line (FSL) rats on cognition and depression and anxiety-related behaviour later in life.

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## 3.2 Abstract and keywords

Major depressive disorder (MDD) also affects children and adolescents, with treatment options currently favouring selective serotonin reuptake inhibitors. Earlier maturation of serotonergic over noradrenergic neurotransmission may explain this phenomenon. The current study investigated the effects of early-life administration with venlafaxine, a serotonin-norepinephrine reuptake inhibitor, on behaviour and cognition later in life in Flinders sensitive line (FSL) and Flinders resistant line (FRL) rats. Pregnant FSL and FRL dams were injected subcutaneously (s.c.) for 14 days with 10 mg/kg venlafaxine (Ven) or saline (Sal) from prenatal day 15 (PreND-15). Postnatally, new-born pups were injected s.c. for 14 days with 3 mg/kg Ven or Sal from postnatal day 3 (PostND03) to PostND17. For both FRL and FSL rats, four treatment groups respectively received prenatal+postnatal administration as follows: Sal+Sal, Ven+Sal, Sal+Ven or Ven+Ven. The forced swim test (FST), locomotor activity test (Digiscan®), novel object recognition test (nORT) and elevated plus maze (EPM) were performed on PostND 21, 35 and 60. Data suggest that treatment of control FRL rats during early-life development does not influence behaviour or cognition later in life. However, Ven treatment of stress-sensitive FSL rats reduced their inherent depressive-like behaviour relative to FRL rats, at PostND60, but not at PostND21 or 35. Locomotor activity was unchanged. Cognition (nORT) and anxiety-like behaviour (EPM) was not affected, as observed at PostND21, 35 or 60. Therefore, stress-sensitive, but not control rats are positively affected in early adulthood (i.e. PostND60) following early-life Ven administration.

***Keywords***

Pre- and postnatal exposure, venlafaxine, Flinder's sensitive line rats, Flinder's resistant line rats, behavioural tests.

# 3.3 Text

## 3.3.1 Introduction

The World Health Organization (WHO) has estimated that major depressive disorder (MDD) will become the second leading cause of disability by the year 2020 in the age group 15-44 years for both genders combined (WHO, 2011). Currently it is estimated that MDD affects 2.8% of children younger than 13, and 5.6% of adolescents between 14 and 18 years of age (Costello *et al*., 2006) and that 25% of children will have experienced a major depressive episode (MDE) by the time they reach adulthood (Kessler *et al*., 2001). Hence, it is clear that MDD has a very strong correlation with onset during childhood and adolescence (Angold *et al*., 1998; Christie *et al*., 1989; Kessler *et al*., 1994; Lahey *et al*., 1996). Even more alarming is the escalation in the prevalence of MDD in this age group, accompanied by increased prescription rates during the last decade (Delate *et al*., 2004; Zito and Safer, 2001; Zito *et al*., 2000; 2002).

The same DSM-IV criteria (American Psychiatric Association, 1994) are used for the diagnosis of MDD in children, adolescents and adults (American Academy of Family Physicians, 2000). However, younger patients have significantly fewer treatment options compared to adults. In Britain, 6 selective serotonin reuptake inhibitors (SSRIs) have been banned as treatment options in patients younger than 18 years (MHRA, 2003), while The Food and Drug Administration (FDA) in the USA has approved fluoxetine as the only antidepressant indicated for the treatment of MDD in children and adolescents (Bylund and Reed, 2007; Wagner, 2005).

The SSRIs, that preferentially target serotonergic neurotransmission, have been demonstrated to be clinically more effective in treating MDD in young patients than the tricyclic antidepressants (TCAs), that preferentially target noradrenergic neurotransmission (Bridge *et al*., 2007; Kratochvil *et al*., 2006; Mason *et al*., 2009). This superior effectiveness of the SSRIs in juveniles has been attributed to the earlier development and maturation of the serotonergic system during this period of development, as compared to the noradrenergic system (Bylund and Reed, 2007). Brain neurodevelopment has been studied in several species, including man, but most extensively in the rat. Of importance is that the age-related patterns for the

development of the monoaminergic systems, regardless of the biomarker measured seem to be similar across species. Consequently it has been possible to delineate much information about neurodevelopment in humans from appropriate animal studies.

In the rat brain, serotonin-containing neurons are the first to be detected in the brain of the 8 mm embryo, whereas the first signs of norepinephrine-containing neurons present days later when the embryo reaches 11 mm in size (Murrin *et al*., 2007). By postnatal day 15 (PostND15), the synaptogenesis of the serotonin system reaches 75% of maximum, compared to the 55% of the noradrenergic system (Lauder and Bloom, 1975). Finally, the serotonergic system reaches maturity in postnatal week 3, the time of weaning, whereas the noradrenergic system reaches this only by the 5th postnatal week, corresponding to the onset of sexual maturity (Murrin *et al*., 2007).

The standard duration of drug treatment of MDD is considered long-term (a minimum of 1-3 years following a first episode) and in the case of younger patients it is recommended that treatment be continued for a period of at least twelve months (Pine, 2002). The impact that such long-term antidepressant exposure can have on the developing brain is additional motivation to investigate the potential long-term neurodevelopmental effects thereof, which may potentially manifest only in adulthood (Andersen and Navalta, 2004). Indeed, are such long term effects benign or beneficial, or are there more sinister effects that adversely affect the individual?

The current study aimed to investigate the long-term effects of early-life administration of venlafaxine, a drug known to modulate both serotonergic and noradrenergic neurotransmission, on depressive-like, anxiety-like and cognitive behaviours later in life. FSL rats were used for this study in order to correlate with humans born with a predisposed increased risk for developing psychiatric disorders such as MDD. The study furthermore aimed to differentiate the effects of chronic administration at two age intervals, namely prenatal and postnatal, and also to determine the age at which drug-induced changes manifests itself most prominently.

## 3.3.2 Materials and methods

### 3.3.2.1 Animals

Male Flinders sensitive line (FSL) rats, and a corresponding behavioural control line, the Flinders resistant line (FRL) rats were used for the current study. Pregnant female dams, as carriers of the unborn male foetuses, were used during the prenatal phases of the study for the administration of drug or vehicle. All animals were housed under conditions of constant temperature (22 ± 1°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.

From the Animal Centre log books from 2009 and 2010, the number of litter per dam was calculated as 8.2 ± 0.3 for FRL rats and 8.5 ± 0.5 for FSL rats, with the nominal difference being statistically insignificant. Furthermore, the sex distribution of male and female pups for FRL rats was calculated as 53.0 ± 1.8% male and 47.6 ± 1.8% female and for FSL as 52.4 ± 1.6% male and 48.2 ± 1.6 female, again with no statistically significant difference (*data not shown*). The data therefore suggest a birth rate of approximately 50% male pups per litter, but with a degree of variance per pregnancy.

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

### 3.3.2.2 Drug treatment

Venlafaxine hydrochloride (a kind gift from Cipla Medpro, Cape Town, South Africa) was dissolved in saline and administered at doses that were used previously to investigate antidepressant-like effects or other central action. As such, pregnant dams were injected subcutaneously (s.c.) with either vehicle control (saline) or venlafaxine at a dose of 10 mg/kg/day (Folkessen *et al*., 2010; Larsen *et al*., 2010; Scaini *et al*., 2010) between 08:00 to 12:00 each morning, for 14 consecutive days from natal days -15 to -1 (i.e. prenatal phase). New-born pups were injected with either vehicle control or venlafaxine at a dose of 3 mg/kg/day s.c. (Dawson *et al*.,

1999) between 08:00 to 12:00 each morning for 14 consecutive days from natal days +3 to +17 (i.e. postnatal phase). In both instances, pregnant dams or new-born pups were measured every day and injected according to the weight on the specific day. Pregnant dams received up to 1.0 ml of either drug or saline, while the new-born pups received a total volume of up to 0.15 ml. Four treatment groups (usually consisting of 8 rats/group, but not less than 3 rats/group) for both FRL and FSL rats received injections prenatal + postnatal as follows: 1. (saline + saline), 2. (venlafaxine + saline), 3. (saline + venlafaxine) and 4. (venlafaxine + venlafaxine). The study aimed to employ 8 rats per treatment group, but due to lower than expected birth rate of male pups in some cases (natural variance), some groups had as little as 3 rats per group.

### 3.3.2.3 Behavioural tests

Following the prenatal and postnatal vehicle/drug treatments, the animals were housed under normal conditions until postnatal days 21, 35 or 60 (PostND21, 35 or 60), at which time the behavioural and memory tests described below were performed. All behavioural tests were performed sequentially between 1 to 4 hours after the start of the dark cycle (i.e. 19:00 – 22:00), implementing 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. The tests were carefully spaced to allow 30 minutes between each test for acclimatisation. 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 verified that preceding tests in the test battery do not significantly affect any of the subsequent tests (*data not shown*).

Following 30 minutes acclimatisation, **cognition** was evaluated by implementing the standard **nORT** test, as described previously (Abildgaard *et al*., 2011). Briefly, the nORT was performed in a square box (dimensions L x W x H = 50 x 50 x 40 cm) with opaque (black) walls and under low light intensity (20 lx). Each test consisted of an acquisition and a retention trial, spaced 90 minutes apart, and with the box wiped clean with 10% ethanol after each trial to eliminate any olfactory cues in subsequent tests. In both trials the animals were placed in the centre of the box facing the objects, with two immovable objects (see below) placed in two corners, 20 cm from

the walls. Animals were left to explore the objects for 5 minutes, during which time the exploration was video-recorded with a camera installed directly above the box. Exploration was defined as sniffing, licking or physically touching an object, and scoring involved the measurement and calculation of the total time spent exploring the familiar or novel object in each trial. In the first (acquisition) trial, the animals were left to freely explore two identical objects (red metal balls, immovably mounted on a flat surface), followed 90 minutes later by the second (retention) trial, during which one of the objects used in the acquisition trial (now familiar object) was replaced with a novel object (green metal squares). Rats tend to spend more time exploring novel objects when memory is intact and therefore the time spent exploring the novel object, relative to the familiar one, is considered to be indicative of memory consolidation.

Following 30 minutes acclimatisation, **locomotor activity** was measured by implementing the **DAAM** apparatus under low light intensity (20 lx), as described previously (Korff *et al*., 2008). Briefly, each animal was placed in a Digiscan® box, with horizontal movement scored as the number of beam breaks as well as the total distance covered during a 5 minute period.

Following 30 minutes acclimatisation, **anxiety-like behaviour** was measured by implementing the standard **EPM** under a light intensity of 80 lx in open arms and 20 lx in closed arms, as described previously (Carola *et al*., 2002). The apparatus consisted of a plus-shaped maze (1 m x 1 m and 10 cm arm width), elevated 50 cm from the floor. Two opposing arms were enclosed with a 30 cm high black opaque wall (designated the closed arms) while the remaining two arms remained open (designated open arms), without ledges. As anxiety-like levels decrease, rats tend to spend more time exploring the open arms, whereas rats tend to spend more time in the closed arms (perceived as more protective) as anxiety levels increase.

The animals were placed in the centre of the maze, facing an open arm and left to explore the maze for 5 minutes. During this time the movement of the animals were recorded with a video recorder installed above the maze. 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.

Following 30 minutes acclimatisation, **depressive-like behaviour** was measured by implementing the standard **FST** under higher light intensity of 200 lx, as described previously (Liebenberg *et al*., 2010). Rats were placed into a cylindrical tank (60 cm high and 30 cm in diameter) containing 30 cm of water maintained at 23°C. The rats were allowed to swim for 5 minutes, during which time swimming behaviour was video-recorded via a camera installed in front of the cylinders. It is important to note that no conditioning swim trial 24 hours prior to the scoring trial is necessary with FSL rats (Overstreet and Griebel, 2004; Porsolt, 1977). Rats were scored as immobile (as opposed to swimming) when only the necessary movements were made to keep their heads above water. Swimming was defined as horizontal movements throughout the swim cylinder that includes crossing into another quadrant, and climbing was defined as upward-directed movements of the forepaws along the side of the swim chamber (Cryan *et al*., 2002).

### 3.3.2.4 Statistical analysis

The unpaired Student's t-test (two-tailed) was performed to compare the data from two treatment groups. Multiple comparisons relative to control were analysed by one way analyses of variance (ANOVA) followed by the Dunnett's post-test. GraphPad Prism® (version 5.00, San Diego California, U.S.A.) was used for all statistical analyses and graphical representation. Data are presented as averages ± S.E.M. with a value of $p < 0.05$ taken as statistically significant.

## 3.3.3 Results

**Early-life administration of venlafaxine to stress-sensitive FSL rats reversed inherent depressive-like behaviour at postnatal day 60, but not earlier, and comparable to that observed in control FRL rats.** *Table 1* displays the time spent immobile during the FST, as observed on postnatal days 21 and 35, while the data from PostND60 is illustrated in *Figure 1*. The data in *Table 1* does not indicate any significant differences in the time spent immobile between the different venlafaxine-treated groups (i.e. pre- and or postnatal) compared to their vehicle-treated control group. It can be seen in *Figure 1(a)* that FSL rats (62.5 ± 15.9) display a higher depressive-like activity relative to FRL rats (28.1 ± 6.9) without venlafaxine treatments (i.e. pre- and postnatal vehicle treated). In *Figure 1(c)* it can be seen that

in FSL rats at PostND60, the depressive-like activity is significantly reduced following treatment with venlafaxine either in the prenatal phase (23.1 ± 7.6) or prenatal and postnatal phases (20.8 ± 6.8), relative to the control FSL rat group (62.5 ± 15.9). This reversal did not reach statistical significance following treatment with venlafaxine in only the postnatal phase (27.5 ± 10.9) (i.e. Sal+Ven group), most likely due to a lack of statistical power (n = 4). Also important, as can be seen in *Figure 1(b),* is that the early-life treatment with venlafaxine did not alter immobility (and consequently, depressive-like behaviour) in FRL rats at PostND60 compared to the FRL controls.

Data from the FST suggested a decrease in the time spent immobile in the FSL drug-treated rats (*Figure 1(c)*), but not in the drug-treated FRL rats (*Figure 1(b)*). This significant decrease in depressive-like behaviour as observed in the FSL rats are supported by the data from the **DAAM**, as **no significant difference in the overall locomotor activity was observed in the FSL rats, compared to the FRL rats following either vehicle- and/or venlafaxine-treatment during pre- and/or postnatal development** (*Table 2*).

**Early-life treatment of venlafaxine to stress-sensitive FSL rats did not significantly affect cognitive function at any age evaluated, as compared to vehicle controls.** *Figure 2* depicts the time spent exploring the novel object in the retention trial by the FSL and FRL rats. It can be seen in *Figure 2(a)* that there was indeed a statistical significant difference between explorative behaviour between the FSL (3.1 ± 0.9) and FRL control rats (8.6 ± 2.1) (i.e. vehicle treated pre- and postnatal). In *Figure 2(c)*, no statistical difference in the explorative behaviour was observed in any of the venlafaxine treated FSL rats (i.e. pre- and/or postnatal) compared to the FSL control rats.

**Early-life treatment of venlafaxine to stress-sensitive FSL rats did not significantly affect anxiety-like behaviour at any age evaluated, as compared to vehicle controls.** *Figure 3(a)* suggests no significant difference in the time spent exploring the open arms of the EPM were observed between the FSL (56.0 ± 9.4) and FRL (56.0 ± 7.6) control rats (i.e. vehicle treated pre- and postnatal). Venlafaxine treatment during pre- and/or postnatal development did not affect the

anxiety-like behaviour of the FSL rats compared to their FSL control group (*Figure 3(c)*).

### 3.3.4 Discussion

The present study used a genetic animal model of depression, viz. the FSL rat, to investigate the long-term effects of the SNRI, venlafaxine, on behaviour and/or cognition later in life, following early pre- and/or postnatal exposure as well as the age at which these effects were most robust.

By targeting both the serotonin and norepinephrine transmitter systems in the juvenile brain, it may be possible to affect the overall development of the brain and its corresponding effects on neurotransmission (Feeney and Westerberg, 1990; Kline *et al*., 1994) and only to observe the resulting effects later in life when maturity is reached (Andersen and Navalta, 2004).

The data from the FST and nORT supported the face validity of the FSL rats as an animal model for depression. Although previous studies have demonstrated increased anxiety-like behaviour in FSL controls compared to FRL controls, following induced stress, it has been suggested that increased anxiety may not be a predominant feature of FSL rats compared to FRL rats under baseline conditions (Overstreet *et al*., 2005). Furthermore Braw and colleagues (2006) indicated that anxiety levels observed in adult FSL rats are not observed in pre-pubertal pups, who display significantly decreased anxiety compared to FRL controls. Following chronic saline administration to the FRL and FSL control rats in the current study, the anxiety-like behaviour of the FSL rats were comparable to that of the FRL control. This is the first time, to the knowledge of the authors, that such behaviour has been reported following chronic stress in the FSL rats.

Data on the long-term safety and efficacy of serotonin targeting antidepressants are numerous, while the data regarding noradrenergic targeting drugs are limited. Treatment with SSRIs during foetal development has suggested no risk for any major malformations (Louik *et al*., 2007), but increased risk of preterm delivery, lower birth weight and pulmonary hypertension in new-born babies still remain a concern (Chambers *et al*., 2006; Rahimi *et al*., 2006). As mentioned the available data on

noradrenergic targeting drugs are very limited, especially in studies investigating the long term effects following chronic pre- and postnatal exposure to these drugs.

As mentioned, the FSL rat is a validated animal model for depression (Janowsky *et al*., 1980; Overstreet *et al*., 1995; Dremencov *et al*., 2004) and presents with a natural increase in immobility time during the FST, compared to the FRL rats (Liebenberg *et al*., 2010). This was confirmed by the current study (*Figure 1*).

The current study identified PostND60 as the age at which the most robust behavioural and cognitive alterations were observed, following chronic treatment with an SNRI (i.e. venlafaxine) during juvenile development (*Table 1* and *Figure 1*). Furthermore the study suggested that early-life treatment with venlafaxine (pre- and/or postnatal) did not significantly alter the anxiety-like behaviour of the stress-sensitive FSL rats or FRL rats, compared to their respective controls (*Figure 3*). However, despite their increased sensitivity to stressful conditions, increased anxiety has not been documented to be a prominent feature of the FSL strain (Neumann *et al*., 2011; Overstreet *et al*., 2005), which may explain the conflicting data of the unaffected anxiety-like behaviour observed in the current study versus the increased anxiety documented in previous studies (Kokras *et al*., 2011).

Regarding memory consolidation of the animals, as measured in the nORT, the current study confirmed the FSL rat as an animal model for depression, since they spent significantly less time exploring the novel object compared to the FRL control group (*Figure 2*). In fact, is has been well established that depressed patients show a general loss of interest in activities of normal life (Günther *et al*., 2004), so that the findings of the current study in in accordance with human behaviour. Chronic administration of venlafaxine during pre- and/or postnatal development did not significantly change this cognitive deficit evident in FSL rats compared to the FSL control group (*Figure 2(c)*). These results are conflicting with previous studies, indicating a significant increase in both spatial and working memory, following chronic venlafaxine treatment to Wistar and albino mice, respectively (Nowakowska *et al*., 2002; Dhir & Kulkarni, 2008; Monleón *et al*., 2008). It is important to notice that the study design (i.e. treatment period and administration route) differed between the current study and previous ones, which may explain the conflicting data.

The most important observation of the current study is that early-life exposure to venlafaxine engenders a significant decrease in the natural depressive-like behaviour in two of the three FSL venlafaxine-treated groups (i.e. prenatal and pre- and postnatal). Interestingly, this was not observed in any of the FRL venlafaxine-treated groups (*Figure 1*). As the reversal of depressive-like behaviour was only observed in the FSL and not in the FRL rats, it may indicate that the animals, more prone to depressive-like behaviour, gain from early-life treatment, without any adverse effects. The more resistant type of animals (i.e. FRL rats) did not present with any advantages or disadvantages when exposed to venlafaxine-treatment during early-life development. This significant decrease of depressive-like behaviour in FSL rats was supported by the data of the locomotor activity of the animals in the DAAM, as no significant difference in the FSL vehicle-treated group (i.e. pre- and postnatal), compared to the FRL vehicle control group was observed (*Table 2*). Previous studies have demonstrated increased locomotor activity later in life, following venlafaxine treatment during early postnatal development (Larsen *et al*., 2010). This is in contrast to the results obtained in the current study, but might be the result of different treatment regimens and administration routes.

Dawson and Tricklebank (1995) suggested that the number of open arm entries may act as a parameter of locomotor activity of the animal and as there were no significant differences in the number of open arm entries in the EPM (*data not shown*), the data of the current study remains valid. In addition, no significant difference in the overall locomotor activity in any of the drug-treated groups (i.e. pre- and/or postnatal), FSL or FRL, were observed on PostND60 (*Table 2*) and thus supporting the decrease of depressive-like behaviour, as it is clear that the increase in mobility was not a result of increased overall locomotor activity of the FSL rats.

In conclusion, early-life venlafaxine administration has favourable effects on the depressive-like behaviour of the stress-sensitive FSL rats later in life i.e. early adulthood. However, this beneficial effect does not extend to alteration in anxiety-like behaviour or improvements of cognitive function later in life. If the data from the study could be directly related to human patients, it would suggest that patients, who have a predisposition to MDD, may benefit from early-life treatment by significantly decreasing natural depressive-like behaviour without altering cognition, anxiety or locomotor activity in adulthood. Future studies, investigating the neurochemical

effects of pre- and postnatal administration as well as other age groups, may further expand our knowledge into the long-term safety, efficacy and adverse effects caused by antidepressant use during early-life development.

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## 3.6 Table captions

**Table 1:** Time spent immobile in a 5 minute forced swim test (FST) by FSL and FRL rats, following vehicle and venlafaxine treatment during the indicated early-life phases. The various early-life treatments include pre- and postnatal vehicle control (Sal+Sal), prenatal venlafaxine + postnatal saline (Ven+Sal), prenatal saline + postnatal venlafaxine (Sal+Ven) and pre- and postnatal venlafaxine (Ven+Ven). The ages when behaviour was measured include postnatal day 21 (PostND21) and 35 (PostND35). A one way ANOVA followed by a Dunnett's posttest was performed to compare the different drug treatment groups with their respective control, while the unpaired Student's t-test (two-tailed) was performed to compare the data of two treatment groups. All data are presented as mean ± S.E.M and a value of $p < 0.05$ was taken as statistically significant. The group sizes are indicated in the table.

**Table 2:** Horizontal activity in a 5 minute locomotor activity test in FSL and FRL rats, following vehicle and venlafaxine treatment during the indicated early-life phases of development. The various early-life treatments include pre- and postnatal vehicle control (Sal+Sal), prenatal venlafaxine + postnatal saline (Ven+Sal), prenatal saline + postnatal venlafaxine (Sal+Ven) and pre- and postnatal venlafaxine (Ven+Ven). Since drug treatment only affected performance in the FST on postnatal day 60 (PostND60), locomotor behaviour was measured only onPostND60. A one way ANOVA followed by a Dunnett's posttest was performed to compare the different drug treatment groups with their respective control, while the unpaired Student's t-test (two-tailed) was performed to compare the data of two treatment groups. All data are presented as mean ± S.E.M and a value of $p < 0.05$ was taken as statistically significant. The group sizes are indicated in the table.

# 3.7 Tables

**Table 1**

| Age | Rat line | Vehicle control (Sal+Sal) | Drug treatment | | |
|---|---|---|---|---|---|
| | | | **Ven+Sal** | **Sal+Ven** | **Ven+Ven** |
| **PostND21** ***(seconds)*** | **FRL** | 19.3 ± 5.5 (*n=7*) | 98.3 ± 29.6 (*n=6*) | 10.0 ± 4.8 (*n=6*) | 15.6 ± 5.1 (*n=8*) |
| | **FSL** | 25.0 ± 9.0 (*n=6*) | 31.3 ± 6.0 (*n=8*) | 13.1 ± 3.8 (*n=8*) | 16.9 ± 3.3 (*n=8*) |
| **PostND35** ***(seconds)*** | **FRL** | 24.0 ± 8.4 (*n=5*) | 60.8 ± 27.7 (*n=6*) | 28.8 ± 11.4 (*n=4*) | 20.0 ± 5.0 (*n=8*) |
| | **FSL** | 41.9 ± 5.7 (*n=8*) | 33.8 ± 6.8 (*n=8*) | 19.3 ± 6.9 (*n=7*) | 24.3 ± 7.8 (*n=7*) |

**Table 2**

| Parameter | Rat line | Vehicle control (Sal+Sal) | Drug treatment | | |
|---|---|---|---|---|---|
| | | | **Ven+Sal** | **Sal+Ven** | **Ven+Ven** |
| **Horizontal activity (*number of beam breaks*)** | **FRL (*PostND60)*** | 2389.0 ± 184.4 (*n=8*) | 2346.0 ± 119.6 (*n=6*) | 1925.0 ± 234.5 (*n=7*) | 2563.0 ± 246.7 (*n=6*) |
| | **FSL (*PostND60)*** | 2305.0 ± 201.0 (*n=8*) | 2508.0 ± 147.2 (*n=8*) | 2020.0 ± 198.8 (*n=4*) | 1987.0 ± 269.4 (*n=6*) |
| **Total distance covered (*cm*)** | **FRL (*PostND60)*** | 1214.0 ± 98.3 (*n=8*) | 1138.0 ± 74.8 (*n=6*) | 945.7 ± 130.8 (*n=7*) | 1296.0 ± 171.3 (*n=6*) |
| | **FSL (*PostND60)*** | 1250.0 ± 127.7 (*n=8*) | 1291.0 ± 86.0 (*n=8*) | 970.3 ± 139.7 (*n=4*) | 1039.0 ± 171.2 (*n=6*) |

## 3.8 Figure captions

**Figure 1:** Time spent immobile in a 5 minute forced swim test (FST) by FSL and FRL rats, following venlafaxine treatment during the indicated early-life phases of development. The various early-life treatments include pre- and postnatal vehicle control (Sal+Sal), prenatal venlafaxine + postnatal saline (Ven+Sal), prenatal saline + postnatal venlafaxine (Sal+Ven) and pre- and postnatal venlafaxine (Ven+Ven). The age when behaviour was measured is only for postnatal day 60 (PostND60). A one way ANOVA followed by a Dunnett's posttest was performed to compare the different drug treatment groups with their respective control, while the unpaired Student's t-test (two-tailed) was performed to compare the data of two treatment groups. All data are presented as mean ± S.E.M and a value of $p < 0.05$ (*) was taken as statistically significant. The group sizes are indicated in the figure.

**Figure 2:** Percentage time spent exploring a novel object in a 5 minute retention trial in the novel object recognition test (nORT) by FSL and FRL rats, following venlafaxine treatment during the indicated early-life phases of development. The various early-life treatments include pre- and postnatal vehicle control (Sal+Sal), prenatal venlafaxine + postnatal saline (Ven+Sal), prenatal saline + postnatal venlafaxine (Sal+Ven) and pre- and postnatal venlafaxine (Ven+Ven). Comparative behavioural data evident on postnatal day 60 (PostND60) are depicted here. A one way ANOVA followed by a Dunnett's posttest was performed to compare the different drug treatment groups with their respective control, while the unpaired Student's t-test (two-tailed) was performed to compare the data of two treatment groups. All data are presented as mean ± S.E.M and a value of $p < 0.05$ (*) was taken as statistically significant. The group sizes are indicated in the figure.

**Figure 3:** Percentage time spent exploring the open arm in a 5 minute elevated plus maze (EPM) test by FSL and FRL rats, following venlafaxine treatment during the indicated early-life phases of development. The various early-life treatments include pre- and postnatal vehicle control (Sal+Sal), prenatal venlafaxine + postnatal saline (Ven+Sal), prenatal saline + postnatal venlafaxine (Sal+Ven) and pre- and postnatal venlafaxine (Ven+Ven). Comparative behavioural data evident on postnatal day 60 (PostND60) are depicted here. A one way ANOVA followed by a Dunnett's posttest was performed to compare the different drug treatment groups with their respective

control, while the unpaired Student's t-test (two-tailed) was performed to compare the data of two treatment groups. All data are presented as mean ± S.E.M and a value of $p < 0.05$ was taken as statistically significant. The group sizes are indicated in the figure.

## 3.9 Figures

**Figure 1**

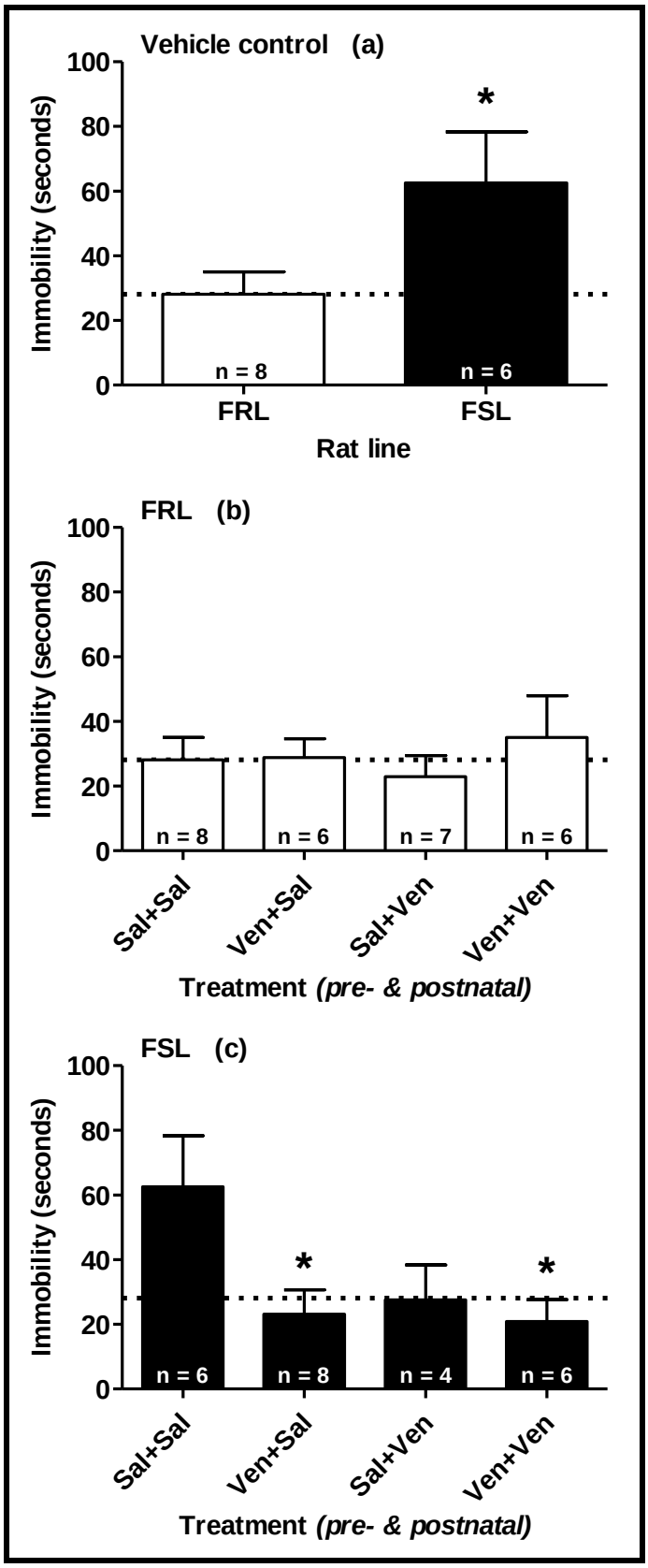


**Figure 2**

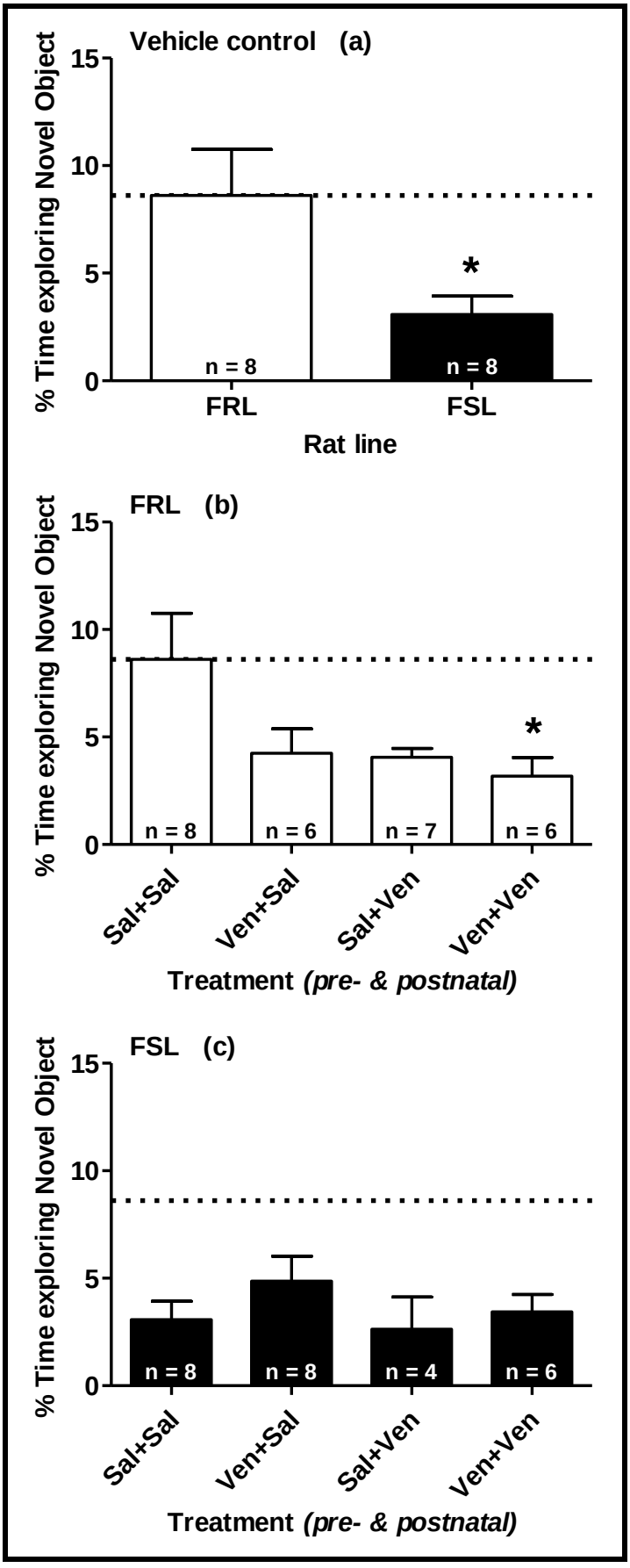
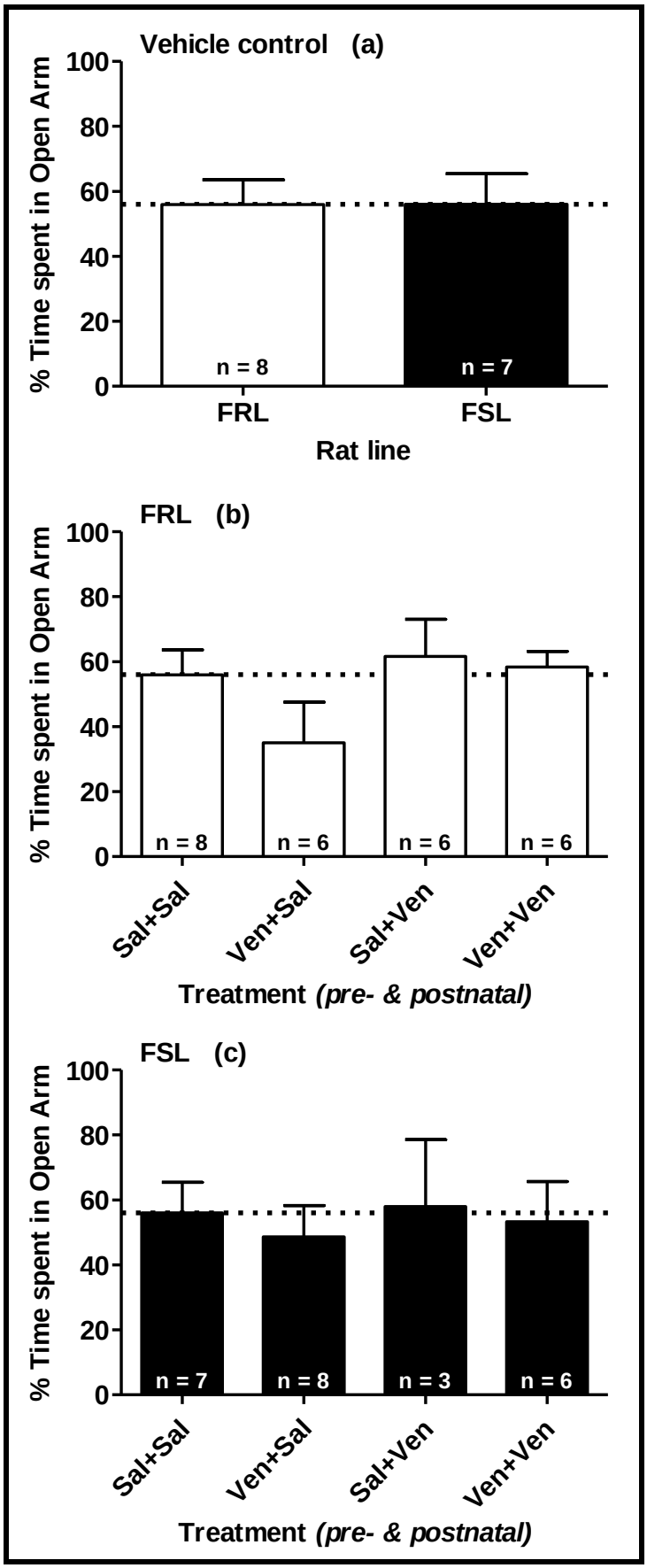

Vehicle control (a)
% Time exploring Novel Object
15
10
5
0
*
n = 8
n = 8
FRL
FSL
Rat line
FRL (b)
% Time exploring Novel Object
15
10
5
0
*
n = 8
n = 6
n = 7
n = 6
Sal+Sal
Ven+Sal
Sal+Ven
Ven+Ven
Treatment (pre- & postnatal)
FSL (c)
% Time exploring Novel Object
15
10
5
0
n = 8
n = 8
n = 4
n = 6
Sal+Sal
Ven+Sal
Sal+Ven
Ven+Ven
Treatment (pre- & postnatal)

**Figure 3**


Vehicle control (a)
% Time spent in Open Arm
100
80
60
40
20
0
n = 8
n = 7
FRL
FSL
Rat line
FRL (b)
% Time spent in Open Arm
n = 8
n = 6
n = 6
n = 6
Sal+Sal
Ven+Sal
Sal+Ven
Ven+Ven
Treatment (pre- & postnatal)
FSL (c)
% Time spent in Open Arm
n = 7
n = 8
n = 3
n = 6
Sal+Sal
Ven+Sal
Sal+Ven
Ven+Ven
Treatment (pre- & postnatal)

# Summary, conclusion and recommendations

Since the results of the current project are presented in two separate sections, namely *Chapter 3* (*Article*) and *Addendum B* (*Additional results*), it was necessary to provide a concise summary of all the results combined. It is the purpose of this chapter to give a comprehensive summary of the results obtained in order to formulate an appropriate conclusion, together with recommendations for prospective studies.

It is important to note that the results discussed herein represent the results obtained from the behavioural tests as performed on postnatal day 21 (PostND21), 35 and 60, following pre- and/or postnatal venlafaxine treatment as well as the comparison of the vehicle control groups (discussed separately). PostND60 represented the most robust effect with regards to all of the behavioural tests and is in accordance with the study objectives discussed in *§ 1.3*. The methods regarding the preparation and performing of specified behavioural tests can be found in *Chapter 3* (*Article*), while background information on these tests can be found in *Addendum A* (*Materials and methods*).

The study aimed to answer the following study objectives as found in *Chapter 1* (*§ 1.3*):

- Validate an appropriate pre- and postnatal treatment regimen with venlafaxine versus vehicle control
- Determine whether chronic venlafaxine treatment during pre- and postnatal development, affects behaviour and/or cognitive function later in life;
- At what age these effects are most robust and
- Whether the stress sensitive FSL rat, a genetic animal model of depression, responds differently to venlafaxine treatment compared to their behavioural control, the FRL rat.

## 4.1 Summary

Firstly, the current study confirmed the treatment regimen used (i.e. dose and administration route) to be safe and effective as no animals died following chronic subcutaneous administration of venlafaxine.

Secondly, the data from the current study indicated that the sex distribution of male and female pups, in both FRL and FSL rats, are approximately 50% with an average litter of eight pups for both rat strains. This suggests that each pregnant FRL and FSL dam will deliver a litter of roughly four male and four female pups on average (*Figure B-1, page 94*).

Finally, *Table 4-1* represents a summary of all of the behavioural tests as observed in the animals tested on PostND60. As mentioned in *§ 3.3.3*, PostND60 showed the most robust changes in the behaviour or cognitive functions of the FSL rats compared to the FRL rats

**Table 4-1: Summary of the behavioural test data performed on postnatal day 60.**

| **Behavioural test** | **Vehicle control FSL vs FRL** | **Drug-treated FRL (*vs FRL control*)** | **Drug-treated FSL (*vs FSL control*)** |
|---|---|---|---|
| **Forced swim test (*depressive-like behaviour*)** | ↓ | ↔ | ↓ |
| **Digiscan® animal activity monitor (*locomotor activity*)** | ↔ | ↔ | ↔ |
| **Novel object recognition test (*cognitive function*)** | ↓ | ↔ | ↔ |
| **Elevated plus maze (*anxiety-like behaviour*)** | ↔ | ↔ | ↔ |

**A summary of the results of the anxiety (EPM), depression (FST) and cognitive (nORT) tests, as well as locomotor tests (DAAM), as observed in the FSL and FRL rats on PostND60, and reported in Chapter 3 and Appendix B. No significant change in behaviour or cognition are indicated as (↔), where (↓) indicates a significant decrease in the designated behaviour or cognitive function, compared to the respective control group. Data were analysed with a one way ANOVA followed by a Dunnett's posttest to compare the different drug treatment groups with their respective controls. An unpaired Student's t-test (two-tailed) was performed to compare the data of two treatment groups.**

The key findings of the current study (*Table 4-1, page 71*) are the following:

1. FSL rats display significant depression-like behaviour, as well as deficits in cognition, compared to FRL control animals. These data constitute robust evidence for the face validity of this model for depression.
2. At PostND21 and PostND35, venlafaxine administration, regardless of whether it was administered pre- or postnatally, did not change depressive-like behaviour, locomotor activity, memory consolidation or anxiety-like behaviour relative to control animals.
3. At PostND60, the stress-sensitive FSL pregnant dams as well as the new-born pup, displayed significantly reduced depressive-like behaviour, compared to their FRL controls, following chronic pre- and/or postnatal venlafaxine treatment. However, anxiety-like behaviour, locomotor activity and cognitive function was left intact.
4. In FRL rats at PostND60, venlafaxine treatment did not change depressive-like, anxiety-like behaviour, or locomotor activity relative to control animals. The cognitive function, however, was significantly decreased in the pre- and postnatal venlafaxine treated groups, compared to the FRL control.
5. In stress-sensitive FSL rats, on PostND60, venlafaxine treatment during the prenatal phase or the pre- and postnatal phase reduced immobility compared to the control FSL rats. The reduction in immobility, following venlafaxine treatment during the postnatal phase did not reach statistical significance, most likely due to a lack of statistical power.

## 4.2 Discussion and conclusion

The current study forms part of an extended project, in which it serves as a pilot for the optimisation of the experimental protocol. Within this context, the aims of the current study was to determine whether early-life treatment (pre- and/or postnatal) with an antidepressant targeting both the serotonergic and noradrenergic systems has any long-term effects on behaviour and cognition later in life, and also to determine at which age these effects become most prevalent.

### 4.2.1 Birth gender ratio

As discussed in *§ 3.3.2.1*, data suggests that there are no differences in the birth gender ratio of FRL compared to FSL rats and also not between sex distributions within each litter (*Figure B-1, page 94*). Although this would suggest that one pregnant dam would deliver approximately four male pups on average, this turned out to vary significantly between dams of the different rat strains, resulting in some test groups of the current study having only three male pups per litter and decreasing statistical power of the data.

### 4.2.2 Behaviour of FSL control rats compared to FRL control rats

Data from FSL and FRL control groups (i.e. chronic pre- and postnatal saline treatment), indicated significant differences in the behaviour and cognition of the two rat strains were observed in the current study. The observations confirmed a number of previously published differences in the traits of FRL and FSL rats, and which led to the FSL rat being regarded well-validated and widely recognised as a stress-sensitive rat line and animal model of depression.

Firstly, the adult FSL rat (i.e. PostND60) displayed significantly higher depressive-like behaviour, compared to the FRL control line, as suggested by the increased time spent immobile during the forced swim test (FST) (*Figure 3-1(a), page 67*). The significantly increased immobility of the FSL rat, as compared to the FRL control line, is in accordance with a number of previous studies (Dremencov *et al*., 2004; El Khoury *et al*., 2006; Liebenberg *et al*., 2010; Overstreet and Russell, 1982; Schiller *et al*., 1992).

Secondly, the FSL control group displayed significantly impaired memory consolidation in the retention trial of the novel object recognition test (nORT), as compared to the corresponding FRL control line (*Figure 3-2(a), page 68*). This natural impairment is in accordance with a previous study (Abildgaard *et al*., 2011; Günther *et al*., 2004) as well as clinical evidence indicating memory impairment in depressed patients.

Finally, previous studies have indicated increased anxiety-like behaviour in the FSL control rats, compared to FRL controls (Kokras *et al*., 2011; Slabbert *et al*., 2010; Liebenberg et al., 2009). However, the data from the current study does not support this finding and our data suggest that FSL rats display anxiety-like behaviour comparable to that of the FRL control (*Figure 3-3(a), page 69*). Importantly, the current study investigated anxiety-like activity in young rats (PostND21, 35 and 60), which may account for this difference. In addition, there are data to suggest that increased anxiety-like behaviour is not a prominent trademark of the FSL rat under baseline conditions (Neumann *et al*., 2011; Overstreet *et al*., 2005) and also that the anxiogenic behaviour is mainly evident in the social interaction test, where the FSL rats spend significant less time interacting with each other, compared to the FRL rats (Overstreet and Griebel, 2004).

From the above mentioned data, it is clear that the FSL rat is a suitable subject with which to investigate the effects of chronic, pre- and/or postnatal, antidepressant therapy, with relevance to similar treatment in depressed individuals. The data supports the validation of the FSL rat as an animal model for depression as well as its functionality in the current study.

### 4.2.3 Age-dependency of the manifestation of venlafaxine-induced effects

The current study revealed a number of differences in the behavioural and cognitive effects of early-life venlafaxine administration as manifested in FSL rats at PostND21, 35 and 60. The most prominent age-related differences were observed in the FST and nORT (*Chapter 3* and *Addendum B*). Whereas no time-dependent differences were observed in FRL rats, the prominent differences observed in stress-sensitive FSL rats suggests that genetic predisposition is an important determinant of drug action and also that the manifestation of venlafaxine-induced effects are dependent on the neurodevelopmental stage of the pre-pubertal and mature animal. The neurodevelopment of the serotonergic pathway is already at mature levels by PostND21, while the noradrenergic pathway, only reaches maturity by PostND35 (see *§ 2.1.5*). Since these effects are most prominent only in early adulthood (i.e.

PostND60), the data also suggest that early-life venlafaxine administration may induce changes in neurodevelopment and in particular the NE system.

Interestingly, pre-pubertal FSL rats (i.e. PostND21) displayed increased locomotor activity, following pre- and postnatal venlafaxine treatment, compared to the vehicle control group (*Figure B-8(b), page 109*). This increase in locomotor activity was, however, not observed in the FSL rats on PostND35 (*Figure B-8(d), page 109*) or 60 (*Figure B-8(e), page 109*), regardless of venlafaxine treatment regimen, suggesting a transient effect following the chronic venlafaxine treatment during the neurodevelopment phases of life. According to a previous study, venlafaxine-treated FSL rats displayed significantly increased locomotor activity, compared to vehicle controls, in a four night open field test observation (Wikell *et al*., 2001). This initial increase in locomotor activity was, however, not observed throughout the remainder of the experiment, supporting our notion of a transient effect of venlafaxine.

Furthermore, the FSL control rats displayed significantly less anxiety-like behaviour than the corresponding FRL controls in the elevated plus maze (EPM) on PostND21 (*Figures B-10(a) and (b), page 113*). However, this was not observed in the FSL controls on PostND35 (*Figure B-10(d), page 113*) or 60 (*Figure B-10(f), page 113*), compared to the corresponding FRL controls (*Figures B-10(c) and (e), page 113*), again suggesting genetic predisposition to play a role, and that the effect is transient. The data reveals a pattern of reduced anxiety levels during pre-puberty in FSL rats, and a return to adult control levels as physiological and neurodevelopmental maturity is reached. This observation is supported by a previous study (Braw *et al*., 2006) which investigated pre-pubertal FSL rats as an animal model for paediatric depression, with specific focus on the anxiety-like behaviour of the animal. As mentioned the data from the current study indicated a significant lower level of anxiety in the pre-pubertal rat, compared to the adult FSL rat. In addition to the transient anxiety effect in the pre-pubertal FSL rats, these animals also displayed comparable depressive-like behaviour to their FRL controls on PostND21 (*Figures B-2(a), page 96*) and 35 (*Figure B-2(b), page 96*), but showed a significant increase in depressive-like behaviour on PostND60 (*Figure B-2(c), page 96*), suggesting that this behaviour might also be a transient effect.

Taking into account that the number of entries into the open arm of the EPM by the FSL rats (regardless of treatment regimen) did not significantly differ from the respective controls on either PostND35 (*Figure B-11(a) and (b), page 114*) or 60 (*Figure B-11(c) and (d), page 114*), this supports findings from the locomotor analysis that the locomotor activity of the FSL rats at the various ages are comparable. Furthermore, the lack of change in locomotor activity supports the claim that the observed changes in the FST reflects changes in depressive-like behaviour and that they cannot be explained by changes in locomotor activity (Dawson and Tricklebank, 1995).

No other significant alterations in behaviour or cognitive functions were observed in the FSL rats on either PostND21 or 35, whereas changes observed in FSL rats on PostND60 are discussed in more detail below.

### 4.2.4 Venlafaxine-induced effects in postnatal day 60 FSL rats

The sections above discussed the differences observed between FSL and FRL control rats, as well as the age-related venlafaxine-induced behavioural and cognitive effects. The current section will now discuss in more detail the venlafaxine-induced effects as observed on PostND60.

Firstly, data from the FST suggests that chronic venlafaxine treatment during pre- and/or postnatal development resulted in a significant decrease in the depressive-like behaviour of the FSL, but not FRL rats, as compared to their respective controls (*Figure 3-1, page 67*). The decrease in depressive-like behaviour did not reach statistical significance in the group that received venlafaxine only during the postnatal stage, which may be explained by the lack of statistical power (n=4). As noted above that this effect is seen in FSL, but not in stressed control FRL rats, suggests the role of genetic predisposition in long-term pharmacological response. Importantly, current models of anxiety-related disorders in humans propose that these disorders result from a combination of environmental factors and genetic predisposition (Kendler *et al*., 1995; 2003; Silberg *et al*., 1999). Hence, one may expect that the effect of drug treatment, as a co-morbid factor to environmental factors, will be different in animals with different genetic predisposition. The results

of the current study were therefore expected and support this environmental-genetic model of depression.

In addition, as mentioned above, the venlafaxine-induced decrease of depressive-like behaviour observed in the FSL rats at PostND60 cannot be explained by changes in locomotor activity. In this regard the Digiscan® animal activity monitor (DAAM) indicated comparable locomotor activity data for all FSL treatment groups (*Table 3-2, page 64*). Previous studies, however, reported a significant increase in locomotor activity following chronic venlafaxine treatment (de Oliveira *et al*., 2004; Kumar *et al*., 2010). Factors that may account for this apparent disparity include the young age of the rats tested in the current study, as well as differences in the treatment regimens (ages and duration) implemented.

Modifications to the FST, as also implemented in the current study, enabled the differentiation of serotonergic and noradrenergic mechanisms in effects observed in the FST (Cryan *et al*., 2002). In this regard, enhanced swimming behaviour is associated with enhanced serotonergic mechanisms, whereas enhanced climbing behaviour is associated with enhanced adrenergic mechanisms. This notion is based on observations with several antidepressants with known effects on these systems. This has also been supported by other studies, where chronic treatment with a tricyclic antidepressant (TCA) significantly increased the time spent climbing, without affecting swimming (Larsen *et al*., 2010), and where chronic treatment with a selective serotonin reuptake inhibitor increased the time spent swimming, without affecting time spent climbing (Liebenberg *et al*., 2010).

According to data from the current study, chronic venlafaxine treatment during pre- and postnatal development significantly increased the climbing action of the FSL rats (*Figure B-7(a) and (b), page 107*), but had no significant effect on swimming (*Figure B-7(c) and (d), page 107*), as compared to the vehicle control. Similar effects were not observed in the corresponding FRL rats. However, a previous study reported that chronic venlafaxine treatment of adult Sprague Dawley rats significantly increases the swimming activity of these animals, without affecting climbing action (Larsen *et al*., 2010). Here it is important to note that the current study employed different administration regimens (i.e. age and duration) and performed behavioural tests at an earlier age. As the climbing, and not the swimming action, of the FSL rats

were affected by chronic venlafaxine treatment in the current study, an increase in the noradrenergic levels is proposed in these animals. This increase in noradrenergic levels do not support the selectivity of venlafaxine (as described in *§ 2.1.7.4*) and requires additional investigation (e.g. neurochemical studies) in order to confirm the current results.

Secondly, as discussed in *§ 4.2.2*, the data from the novel object recognition test (nORT) suggested that memory consolidation of FSL control rats is impaired as compared to FRL controls. Following venlafaxine treatment during pre- and postnatal development, memory consolidation was impaired in FRL (*Figure 3-2(b), page 68*), but not in FSL rats (*Figure 3-2(c), page 68*), relative to their respective vehicle controls. The data therefore suggest that early-life venlafaxine treatment does not impair memory consolidation in stress sensitive animals, again supporting the role of genetic predisposition in drug effects. However, the memory impairment, as observed in the pre- and postnatal venlafaxine treated FRL rats, may suggest different drug effects in different pathophysiological subjects (i.e. FRL versus FSL).

Finally, the EPM data in current study did not reveal any venlafaxine-induced changes in the anxiety-like behaviour of FRL or FSL rats, as compared to their respective vehicle controls, regardless the age of the EPM test (*Figures 3-3(b), (c), page 69, B-10(e) and (f), page 113*). Literature reports are not conclusive in this regard, since several studies reported no changes in anxiety-like behaviour following chronic venlafaxine treatment (de Oliveira *et al*., 2004; Larsen *et al*., 2010), whereas others observed a significant increase following treatment with a tricyclic antidepressant and selective serotonin reuptake inhibitor (Kokras *et al*., 2011; Slabbert *et al*., 2010). Also important is that in these studies and the study design differed with regards to the duration and age of administration, as well as the ages of the animals at the time of testing.

In conclusion, the pre- and postnatal administration of venlafaxine to stress-sensitive rats did not significantly affect the anxiety or cognition of these animals. Treatment with the serotonin-norepinephrine reuptake inhibitor, venlafaxine, did however significantly decrease the depressive-like behaviour of these stress-sensitive rats in adulthood (i.e. PostND60), but not at any of the earlier stages of life (i.e. Post21 and 35) compared to their control counterparts (i.e. FRL rats).

Regarding the secondary objectives, as described in *§ 1.3*, the following conclusions were made:

1. The pre- and postnatal treatment regimen used in the current study has been confirmed to be safe and effective, since no disproportionate discomfort, malformations or any casualties were observed throughout the study.
2. Secondly, behavioural changes following early-life administration of venlafaxine were found to be most robust at a later stage in life (i.e. PostND60). Future studies should investigate the effects of these drugs beyond PostND60 in order to confirm and elaborate on the current data. It seems that ages earlier than PostND60 does not present with such major and significant changes in behaviour. Future studies investigating neurological biomarkers associated with the age-related behavioural changes, may provide additional insight into the neurodevelopmental effects underlying these observations.
3. Finally, it was confirmed that the FSL rats responded differently to pre- and/or postnatal treatment with venlafaxine than their control counterparts, the FRL rats. The only effect caused by the venlafaxine treatment in the FRL rats, were the decreased memory consolidation of the pre- and postnatal treated rats, compared to the control rats. This observation needs to be further investigated and may be that venlafaxine decreases the exploratory behaviour in FRL animals with no further decrease in FSL.

Taking all of the abovementioned facts into account, it seems that animals (and potentially humans) with a predisposition to depressive-like behaviour might benefit from early-life treatment with a serotonin and norepinephrine targeting drug such as venlafaxine. Data suggest that this treatment may reverse the congenital depression, without any other overt behavioural side-effects later in life. It remains important to keep in mind that this conclusion is based on the current study in rodents. The study was further limited by a small n-value and needs to be investigated further.

## 4.3 Recommendations

Whereas the current study successfully addressed the study objectives defined in *Chapter 1*, there were a number of limitations to the study, and accordingly a number of recommendations that can be made for future studies.

1. The number of animals employed in the study was in some instances too small and this needs to be addressed to enhance the statistical power of the current findings. Significant variations in litter sizes and sex distribution may be expected (*Figure B-1, page 94*) and it would be advisable to budget for more pregnant dams per test group than predicted by the average birth rate.
2. The current study did not attempt to correlate behavioural data with any biomarkers in the brain. Brain tissue is available to do this in future studies and it may be valuable to determine in the cortex and hippocampus effects on brain-derived neurotophic factor (BDNF) (i.e. biomarker of neuroplasticity), monoamine and metabolite levels, as well as the expression of cGMP, phosphodiesterase type 5 (PDE 5), monoamine transporters and monoamine oxidases. The P-gp protein has recently been highlighted as an important factor in the pharmacokinetics and –dynamics of venlafaxine and may explain differential therapeutic effects in patients. These biomarkers will also assist to unravel the neurobiological mechanisms underpinning the long-term anxiolytic effects of the early-life administration of venlafaxine as well as the altered climbing, but not swimming, action observed in the FSL rats.
3. The study did not investigate the effect of venlafaxine administration at any age beyond 60 days, so that it is not known whether the venlafaxine-induced changes persist into old age. It may be valuable to investigate this possibility in future studies.
4. The current study investigated the effect of venlafaxine administration during pre- and postnatal phases, but not at ages PostND19 to 40, a phase also associated with neurodevelopment. It may be valuable to investigate antidepressant administration also during these phases in future studies.
5. The current study investigated only one antidepressant i.e. venlafaxine. Other classes of antidepressants, targeting the different neurotransmitters systems (i.e. serotonin, norepinephrine and dopamine, respectively), as well as the atypical antidepressants, may also be investigated. This will clarify whether

the effects seen with venlafaxine are class-specific, and also to identify which neurotransmitter and other systems are most likely involved in the observed effects.

6. Furthermore, it would be important for future studies to investigate the effects of the two enantiomers of venlafaxine separately. Isoforms are known to display differential pharmacodynamics and pharmacokinetic profiles, including differences in receptor affinities.

# Materials and methods

This addendum describes all the materials and methods used in the current study. Where these have already been described in *Chapter 3*, reference to the appropriate paragraph numbers is provided, with additional detail where appropriate.

## A.1 Animals

As already described in *§ 3.3.2.1*, the animals used in the current study consisted of Flinders sensitive line (FSL) rats and their behavioural control Flinders resistant line (FRL) rats.

### A.1.1 The Flinders sensitive line rat as an animal model of depression

The FSL rat line was initially bred by the Overstreet laboratory from Sprague Dawley rats to display hyper-cholinergic activity (Overstreet *et al*., 1984), and thereby to be resistant to the toxic effects of the organophosphates, in particular the irreversible cholinesterase inhibitor diisopropyl fluorophosphate (DFP) (Overstreet *et al*., 1979; Russel *et al*., 1982). The Overstreet team co-incidentally observed that these rats display depressive-like behaviour, followed by validation of the FSL rat line as a genetic (inbred) animal model of depression (Janowsky *et al*., 1980; Overstreet *et al*., 2005), and supported by reports from other laboratories across the globe (Liebenberg *et al*., 2010; Neumann *et al*., 2011). It is important to note that the FRL, consequently, are not more resistant to DFP compared to control rats from other breeds, but relative to the FSL strain (Overstreet *et al*., 1979; Russell *et al*., 1982).

Validation of animal behavioural models of human disease has to comply with criteria defined more than forty years ago by McKinney and Bunny (McKinney and Bunny, 1969), including that the 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 hyper-cholinergic characteristics of FSL rats have been postulated to underlie the depressive-like behaviour of these rats, which correlate with the hyper-cholinergic super-sensitivity model of depression (Janowsky *et al*., 1980; 1994) (*§ 2.1.6.2*). This hypothesis provided the first evidence of construct validity of the model. Later studies suggested that the FSL rat line also presents with additional neurobiological correlates with the human brain of depressed patients, such as modulated NO/cGMP signalling following stress (Harvey et al., 2006), 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). Importantly, the altered immobility of FSL rats were normalised by chronic (but not acute) antidepressant treatment (Zangen et al., 1997; Overstreet, 1993; Overstreet *et al*., 1995; Dremencov *et al*., 2004), suggesting predictive validity of the model.

In addition, FSL rat line presents with retarded psychomotor activity (Overstreet and Russel, 1982; Overstreet, 1986), reduced appetite and weight gaining (Overstreet, 1993; 2002), alternated sleep patterns and immune abnormalities (Overstreet *et al*., 2005) and 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) resulting in the conclusion that anxiety might not be a prominent feature of the FSL strain (Neumann *et al*., 2011; Overstreet *et al*., 2005). All of these observations correlate with the depression in humans and provide face validity to the model.

In conclusion, in line with the criteria of McKinney and Bunny mentioned above (McKinney and Bunny, 1969), the FSL rat line can be considered a robust, validated animal model of depression, with face-, predictive- and construct validity, and as confirmed by different laboratories via objectively measurable parameters.

### A.1.2 Limiting the study to male rats only

The hormonal cycles of female rats are known to affect behaviour, which may complicate the interpretation of data following interventions. In the current study, animal behaviour was measured on postnatal day 21 (PostND21), 35 and 60, following early-life treatments. In this regard, female rats become sexually mature at about five weeks of age (Murrin *et al*., 2007; Zeinoaldini, 2005) and vaginal opening takes place around thirty two and thirty four days of age, whereas the first sexual cycle occurs between thirty five and thirty seven days after birth (Moguilevsky *et al*., 1995). These physiological changes represent good physiological markers for the onset of puberty which roughly corresponds to early adolescents in humans (Murrin *et al*., 2007; Zeinoaldini, 2005). Therefore, the inclusion of female rats may influence the behavioural tests of the current study, so that only male rats were included.

## A.2 Drug

The venlafaxine hydrochloride, as used in the current study, was a kind gift from Cipla Medpro, Cape Town, South Africa. It is described chemically as:

*(R/S)-1-[2-(dimethylamino)-1-(4 methoxyphenyl)ethyl] cyclohexanol hydrochloride*

or

*(±)-1-[a [α- (dimethylamino)methyl] p-methoxybenzyl] cyclohexanol hydrochloride, empirical formula of* $C_{17}H_{27}NO_2 \cdot HCl$.

The pharmacological properties of venlafaxine is discussed in *§ 2.1.7.4*.

### A.2.1 Administration and dosage

Rat foetuses and new-born pups are vulnerable to injection injury, particularly with chronic intraperitoneal (i.p.) administration to either the pregnant dam or the pup, so that alternative routes had to be explored. A previous study showed that the bio-availability following s.c. administration was only 2-3% lower than that following i.p. administration (Wright and Wilson, 1983). Therefore, i.p. and s.c. administration

routes can be considered to yield comparable drug levels, but with a reduced risk of injection injury. This similarity in bioavailability enables the use of data from studies implementing i.p. administration to determine a suitable dose for s.c. administration.

Accordingly, all treatment regimens in the current study involved prenatal s.c. administration to pregnant dams as well as s.c. postnatal to new-born pups, both for two weeks (i.e. 14 days). Animals received treatments prenatal:postnatal as follows: saline:saline (control), saline:venlafaxine, venlafaxine:saline or venlafaxine:venlafaxine, as discussed in *§ 1.4*.

The pregnant dams were injected with 10 mg/kg s.c. venlafaxine (Folkessen *et al*., 2010; Larsen *et al*., 2010; Scaini *et al*., 2010), whereas the pups received 3 mg/kg (s.c.) (Dawson *et al*., 1999).

### A.2.2 General housing protocol

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, coined prenatal day 21 (PreND-21). The first series of daily treatment was started one week after the day of conception (i.e. PreND-15) and ended fourteen days later (i.e. PreND-01).

After birth a random selection of four new-born male pups were made from the litter of appropriately treated dams and postnatal treatment was started on postnatal day 3 (PostND03) and ended fourteen days later (i.e. PostND17). The male subjects were weaned on PostND21 and housed under normal conditions (see *§ 3.3.2.1*) in cages of four rats of the same treatment group until the day of behavioural testing i.e. PostND21, 35 or 60.

## A.3 Background and methods for the behavioural tests

A battery of behavioural tests (discussed and described in *§ 3.3.2.3*) were carried out in order to evaluate the effects of the different treatment regimens on depressive-like

behaviour, locomotor activity, cognition and anxiety-like behaviour. Additional background and method information for each test is given below. It is important to note that each behavioural test was separated by an acclimatization period of thirty minutes in order to minimize stress in the animals, caused by moving the cages from one room to another.

## A.3.1 The forced swim test

The forced swim test (FST) was originally developed in the 1970's to detect antidepressant-like behaviour in rats (Porsolt *et al*., 1977), but was later modified in order to distinguish between serotonergic and noradrenergic mechanisms (Lucki, 1997) involved in drug action.

The FST is a very robust and 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. 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, following initial escape-directed movements, develop an immobile posture when exposed to an inescapable cylinder of water twenty four hours later. If an antidepressant drug is administered in an acute regimen to these animals following a first conditioning trial, they persist in engaging escape-directed movements in the second trial for longer periods of time, compared to the vehicle-treated controls (Liebenberg *et al*., 2010). Importantly, FSL rats display inherent depressive-like behaviour, so that a conditioning trial may be omitted (Porsolt *et al*., 1977; 1978).

Four forced swim test cylinders were filled with 30 cm deep water, maintained at 23$^{o}$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. At the end of the seven minutes, each rat was removed from the cylinder and dried with a paper towel and returned to its home cage. Each cylinder was washed with 10% ethanol solution 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 mechanisms of antidepressant action. In this scenario, adrenergic mechanisms will increase climbing, whereas serotonergic mechanisms will favour swimming behaviour (Cryan *et al*., 2002) (*Figure A-1*). The first and last minute of the seven minutes spent swimming, was omitted from the final results, which meant that a total of five minutes was scored in order to maximise the accuracy of the results.

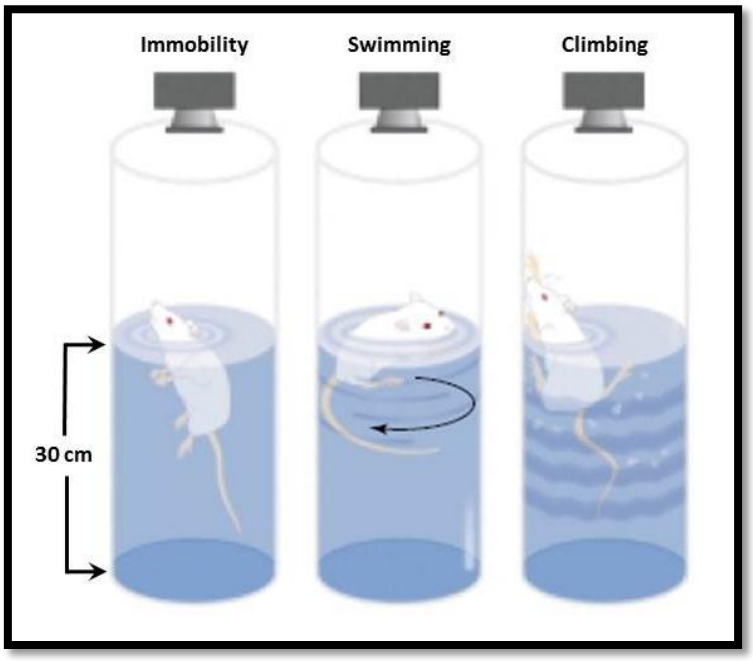



**Figure A-1: Illustration of the swimming, climbing and immobility behaviour of the FRL and FSL rats during the forced swim test, as implemented in the current study (Cryan *et al*., 2002).**

- Immobility is defined as in the traditional Porsolt test (Porsolt *et al*., 1977), when no active movements are made, except those that are necessary 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).

## A.3.2 The Digiscan® animal activity monitor

The Digiscan® animal activity monitor (DAAM), illustrated in *Figure A-2* provides automated and continual computerized monitoring of animal movement. It is more sensitive than visual observation and without the risks of investigator bias (Sanberg *et al*., 1983; 1987).

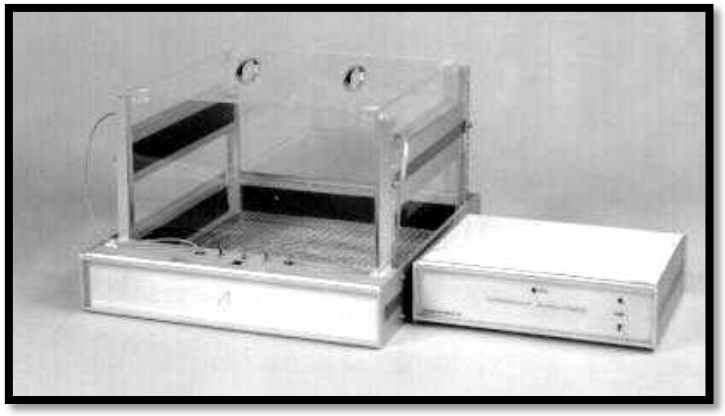


**Figure A-2: A photo of the Digiscan® animal activity monitor, as implemented in the current study.**

The DAAM cages have a series of cross-sectional horizontal infrared light beams at ground level plus additional beams 10 cm above the first. These infrared beams enable the computerized collection of all locomotor activity, to provide a dynamic picture of all the aspects of the animal's horizontal and vertical movement (locomotor activity) throughout the observation period. By breaking any one of the beams, the action is interpreted as an activity score, whereas the breaking of two or more consecutive beams is recorded as a movement score (Korff *et al*., 2008).

In the current study horizontal activity (number of beam breaks) and the total distance covered (measured in centimetres) during the five minute trial were recorded. Each rat was placed in the centre of a separate DAAM cage and left to explore for the five minutes in which the mentioned parameters were measured.

## A.3.3 The novel object recognition test

The novel object recognition test (nORT) was first described over twenty years ago (Ennaceur and Delacour, 1988) and has been used ever since to examine animal memory performance. The test has been used in over a thousand behavioural studies since 1988 and in different variations of the original one. The popularity of the test might be because of the one major advantage that the test has i.e. no aversive or stressful stimuli is needed for the test to be carried out (Rutten *et al*., 2008) (*Figure A-3*).

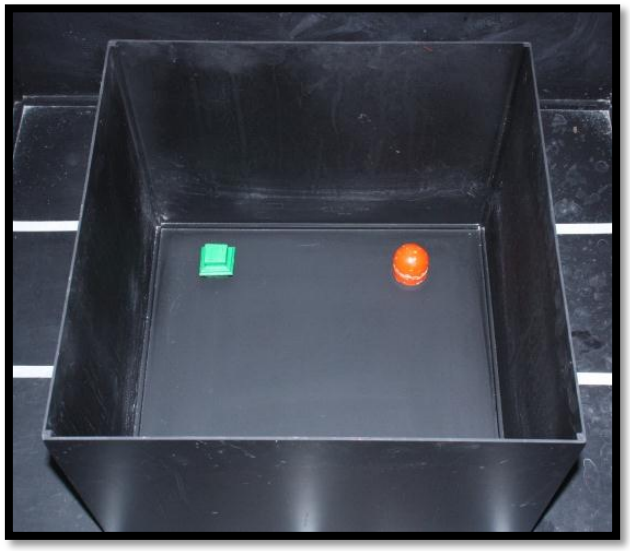


**Figure A-3: A photo of the novel object recognition test box, as implemented in the current study. The photo illustrates the four opaque walls as well as the two different immovable objects.**

The memory performance of the nORT is based on the natural tendency of the animals to explore novel objects relative to a familiar one (Rutten *et al*., 2008) so that impaired memory function would be observed in a rat spending less time exploring the novel object, compared to the familiar one during the retention trial.

In the current study, each rat was placed in a separate nORT box following a thirty minute acclimatization period. During the acquisition trial, the rat was left to explore the nORT box with two identical red objects (see *Figure A-3 (right)*) for a total of five minutes. After the acquisition trial, the rats were returned to their cages and left for ninety minutes before returning for the retention trial. During the retention trial, one

of the red objects was replaced with a novel green object (see *Figure A-3 (left)*). Each rat was again left to explore the nORT for five minutes in which the time spent exploring the familiar and novel object, respectively, was scored.

## A.3.4 The elevated plus maze

The elevated plus maze (EPM) is one of the most widely used animal models of anxiety (Estanislau *et al*., 2011) and has featured in more than nine hundred published articles (Bertoglio and Carobrez, 2000). The test was developed in order to identify anxiolytic and anxiogenic effects of drugs, but has also been used to study long-term behavioural change, such as following early life manipulations (Estanislau and Morato, 2005; Hinojosa *et al*., 2006).

The EPM was first used in the late 1980's by Handley and Mithani in a study which investigated the involvement of the noradrenergic systems in anxiety (Handley and Mithani, 1984). The standard test consists of a cross-shaped apparatus with two enclosed (i.e. walled) and two open (i.e. unwalled) arms (*Figure A-4*).


**Figure A-4: A photo of the elevated plus maze, as implemented in the current study. The photo illustrates the plus-shaped platform, elevated from the floor surface as well as the two enclosed arms.**

The test is based in the natural tendency of the animals to seek the shelter of closed spaces, but also a curiosity-driven tendency to explore the open areas. The balance of time spend in shelter versus exploration is altered by the state of anxiety, and hence also by anxiolytic and anxiogenic drugs (Pellow *et al*., 1985).

In the current study, each rat was placed in the centre of separate EPMs, facing one of the open arms. The rats were left to explore the EPM for five minutes in which the time spent exploring the open and closed arms, respectively, was scored and analysed. After each test, the EPM was wiped cleaned with a 10% ethanol solution in order to eliminate any olfactory cues which would influence the next group of rats.

# Additional results

This addendum contains additional data, not presented in *Chapter 3 (Article)*, and acts as supporting material to the main study. The main study objective of the current study (see *§ 1.3*) was to investigate behavioural and cognitive changes in stress-sensitive Flinders sensitive line (FSL) rats later in life following early-life exposure to the serotonin-norepinephrine reuptake inhibitor (SNRI), venlafaxine (pharmacological action discussed in *§ 2.1.7.4)*. The FSL rat and its behavioural control, the Flinders resistant line (FRL) rat, were used in the current project in order to investigate the long-term effects of an SNRI on subjects with a predisposed natural tendency towards depressive-like behaviour (i.e. FSL), compared to its control (i.e. FRL).

Subjects received venlafaxine during early pre- and postnatal phases of life, following four different treatment regimens (see *§ 1.4*) and subjected to a battery of behavioural tests (*§ 1.4*) on three different ages later in life, i.e. postnatal days 21 (PostND21), 35 and 60. These specific ages represent different stages of development (see *§ 1.3*).

The key results obtained from the behavioural tests are described and discussed in *§ 3.3.3* and *§ 3.3.4*. A summary and in depth discussion, along with the conclusion of the current study, can be found in *Chapter 4* (*Summary, discussion and conclusion*). This addendum contains necessary data relating to pilot studies that were a prerequisite before initiating the main study described in *Chapter 3* as well as supporting data to that already discussed. These pilot studies were designed to address the following questions of relevance for the primary study:

1. Determine what the average litter size and sex distribution of each litter, for both FRL and FSL pregnant dams were, in order to accurately determine the number of pregnant dams needed for the study to ensure that a sufficient number of male pups would be delivered.
2. Compare behaviour and cognitive function of male FRL and FSL control rats on the different, specified days (i.e. PostND21, 35 and 60), following chronic pre- and postnatal saline administration in order to act as a positive control to the venlafaxine treated groups.

The results presented in this addendum compliments the data already presented in *Chapter 3* (*Article*), and will be discussed in more detail within the context of the overall study in *Chapter 4*.

## B.1 Determining the size and sex distribution of each litter

Before the main study could be initiated, as discussed in *§ 1.3, § 1.4* and *§ 3.3.2.1*, it was necessary to first establish litter size and sex distribution per litter that would be required in order to enable an accurate prediction of the number of pregnant dams needed to produce enough male pups for behavioural testing later in life that would limit the project to male rats only (discussed in *§ A.1.2*).

Size and sex distribution per litter of FRL and FSL rats, were obtained from the log books from 2009 and 2010 of the Animal Centre and are briefly discussed in *§ 3.3.2.1*, but are illustrated and discussed in *Figure B-1 (page 94)* below.

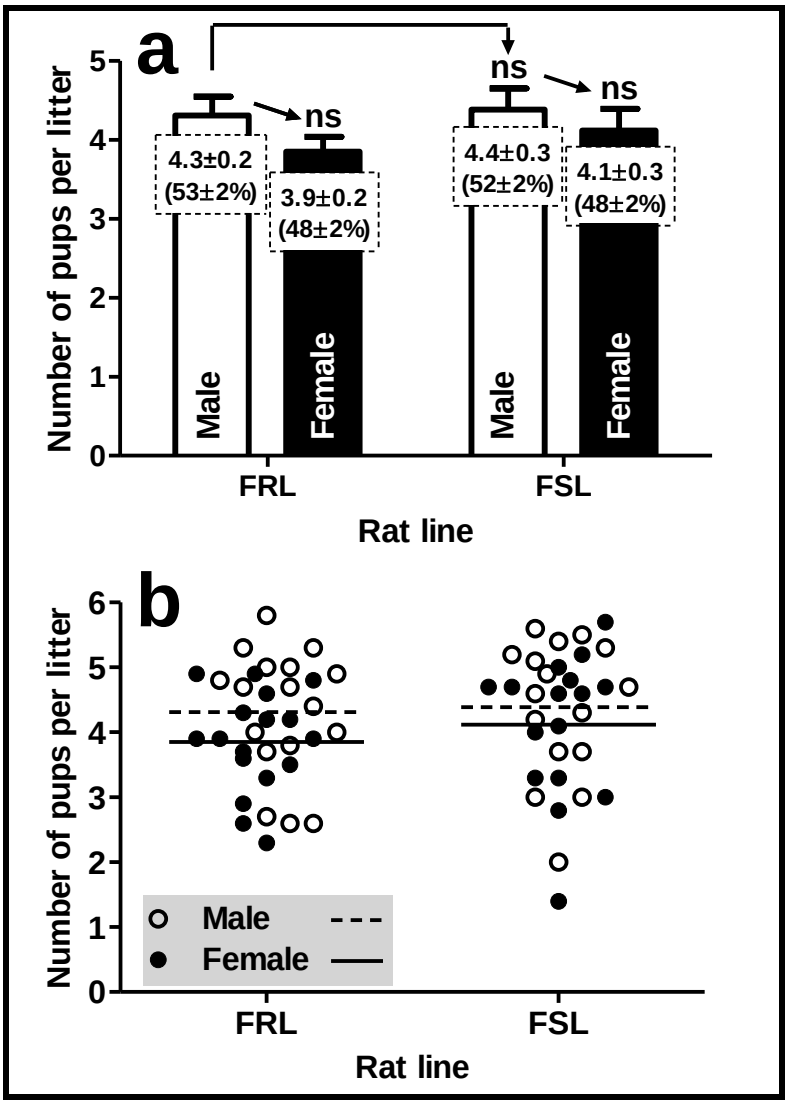



**Figure B-1: Number of pups per litter and sex distribution of FRL and FSL rats.**
**(a) Data are represented as averages ± S.E.M and were captured from data on 173 litters from 17 months for FRL and 219 litters from 16 months FSL rats, as reported in the log books of the Animal Centre for 2009/2010. A two-way ANOVA analysis of the data confirms the absence of an interaction between the rat line (FRL/FSL) and sex (male/female), (F [1, 62] = 0.1549, p = 0.6953), thereby allowing further analysis of the data by a one-way ANOVA followed by the Tukey-Kramer post-test for multiple comparison. (b) A scatter plot of the data clearly indicates the distribution of monthly birth rates as observed during the 17 or 16 month period for FRL or FSL rats, respectively.**

*Figure B-1(a)* illustrates the number of male and female rats of FRL and FSL rats, respectively. As can be seen the number of male versus female pups per litter did not differ significantly for either FRL or FSL rats, and also the number of male rats per litter was not significantly different between FRL and FSL rats. It was therefore concluded that the sex distribution was on average 50:50 and that approximately

four male pups could be expected from each litter. *Figure B-1(b)* (*page 94*) illustrates the distribution of monthly birth rates, as observed during the seventeen or sixteen month period for FRL or FSL rats, respectively. It can be seen that the monthly birth rates for male pups varied from as low as 2 per litter to as high as 5.8 per litter within a particular month, suggesting that much lower than expected birth rates should be foreseen.

## B.2 FRL versus FSL rats (control groups)

Before the main study could be initiated, as discussed in *§ 1.3* and *§ 1.4,* as well as in *Chapter 3*, it was necessary to first establish the baseline behavioural data of the animals for the various behavioural tests to be applied, viz. the FST, locomotor activity, nORT and the EPM. Following pre- and postnatal administration of saline (control) to both FRL and FSL groups, the above behavioural tests were performed on the specified days later in life (i.e. PostND21, 35 and 60) in order to obtain a control value to which the venlafaxine treated groups could be compared to.

Some of the data below have already been reported in table format in *§ 3.3.3*. Furthermore, the results given below also include data not reported in *Chapter 3* (*Article*), but are presented in order to enable a more comprehensive analysis of observations in the FSL rat as a validated animal model for depression (discussed in *§ A.1.1*).

### B.2.1 The forced swim test

*Figure B-2* (*page 96*) depicts the time spent immobile during the forced swim test (FST) of the FSL control group, compared to the FRL control on the different specified ages postnatal.

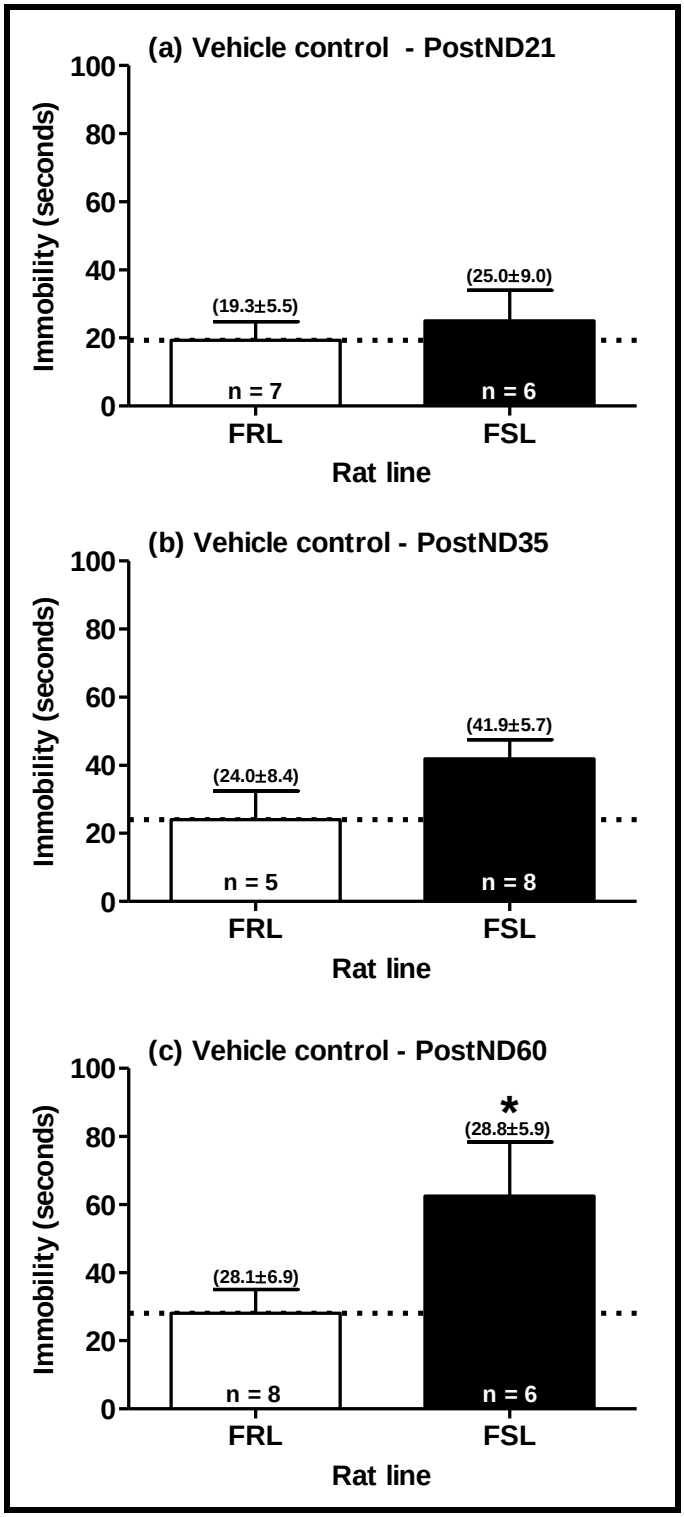



**Figure B-2: Time spent immobile during the FST by the FRL and FSL control groups on the different specified ages postnatal.**
**FRL and FSL control groups at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with the unpaired Student's t-test (two-tailed), with statistical significance taken as $p < 0.05$ (*).**

At postnatal day 21 (PostND21) and 35, the FSL control rats, did not show any significant depressive-like behaviour, as compared to their FRL controls (*Figure B-2 (a* and *b), page 96*). However, at PostND60, the FSL control rats displayed a significantly higher level of depressive-like behaviour, compared to the FRL control group. The data therefore suggest that under our conditions of study the natural increased depressive-like behaviour of FSL rats only presents later in life on PostND60 and not earlier. This observation is in accordance with already published data, suggesting that pre-pubertal FSL rats do not display the same increased depressive-like behaviours observed in adult FSL rats (*§ 4.2.3*), and thus needs to be taken into consideration when designing a study where the effect of drug treatment on behaviour in the FST will be assessed at certain stages of development. These data then confirm that the stress-sensitive FSL rats only displayed significantly increased immobility at PostND60 and not at pre-pubertal ages (i.e. PostND21 and 35) and may indicate a transient effect of increased depressive-like behaviour as maturity is reached.

## B.2.2 The Digiscan® animal activity monitor

Locomotor activity may affect the interpretation of immobility data from the FST. A decrease in locomotor activity may, for example, increase immobility, whereas an increase in locomotor activity may decrease immobility. Changes in immobility in the FST will then not reflect depressive-like behaviour, but locomotor activity. When immobility is unchanged, when increased locomotor activity is associated with increased immobility (i.e. decreased mobility), or when decreased locomotor activity is associated with decreased immobility (i.e. increased mobility), locomotor activity cannot explain any change in immobility, rendering the FST data useful for interpretation as parameter of depressive-like behaviour.

Before the main study could be initiated, as discussed in *§ 1.3* and *§ 1.4* and in *Chapter 3*, it was necessary to establish the general locomotor activity of the FSL and FRL control groups at the specified ages that will be considered in the main study. These data are presented and discussed in *Figure B-3* (*page 98*), below.

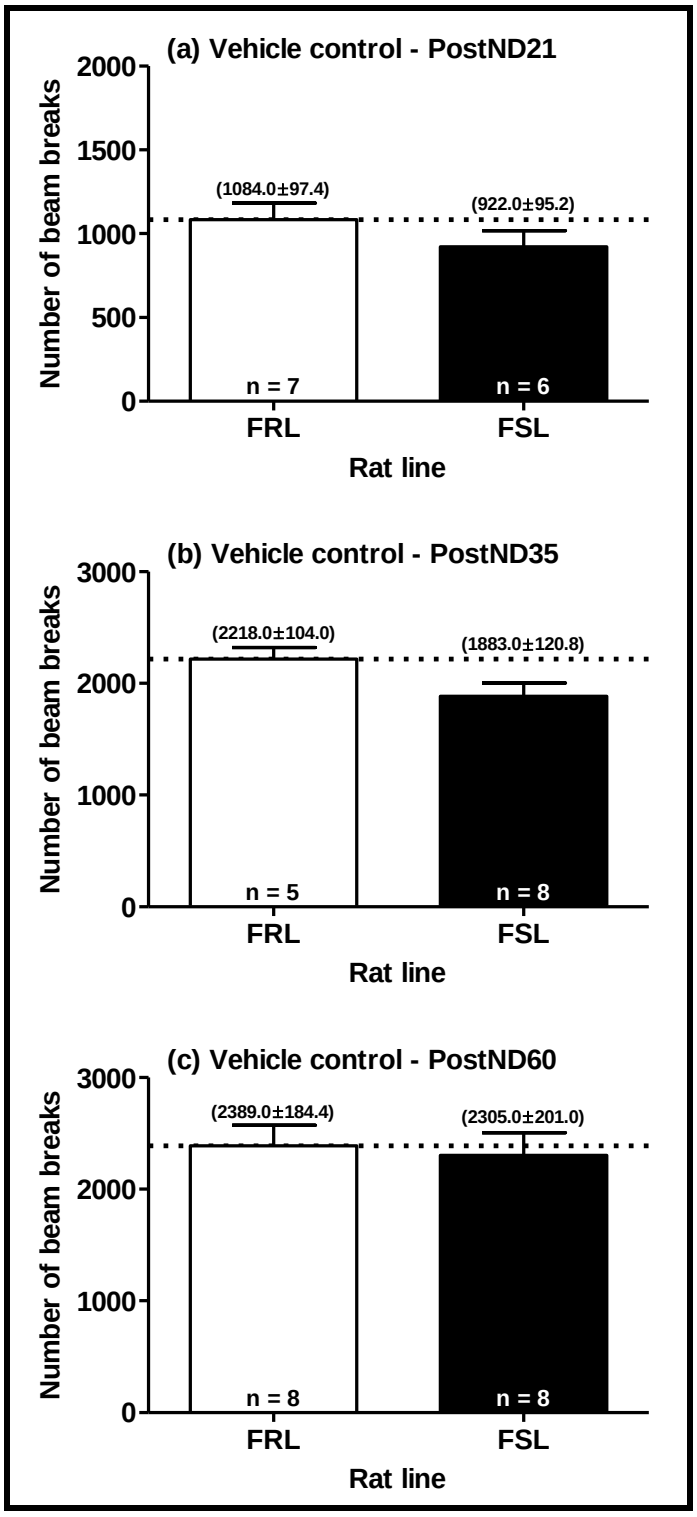



**Figure B-3: Number of beam breaks in the Digiscan® animal activity monitor by the FRL and FSL control groups on the different specified ages postnatal.**
**FRL and FSL control groups at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with the unpaired Student's t-test (two-tailed), with statistical significance taken as $p < 0.05$.**

From the data presented in *Figure B-3* (*page 98*) it can be seen that locomotor activity is comparable in FRL and FSL control rats at PostND21, 35 and 60. The fact that locomotor activity for both the FRL and FSL controls were comparable at all three specified ages of development suggests that any changes in locomotor activity subsequent to venlafaxine treatment cannot be attributed to inherent differences in general locomotor activity between FSL and FRL rats. This study has therefore provided valuable reference data to which the venlafaxine treated groups can be compared to in *Chapter 3*.

## B.2.3 The novel object recognition test

Thirdly, the general levels of memory consolidation (a measure of cognition) of the FRL and FSL control groups at the three different stages of development was undertaken. These data were generated by measuring the extent of memory consolidation in the retention trial of the novel object recognition test (nORT). These data are illustrated below in *Figure B-4* (*page 100*).

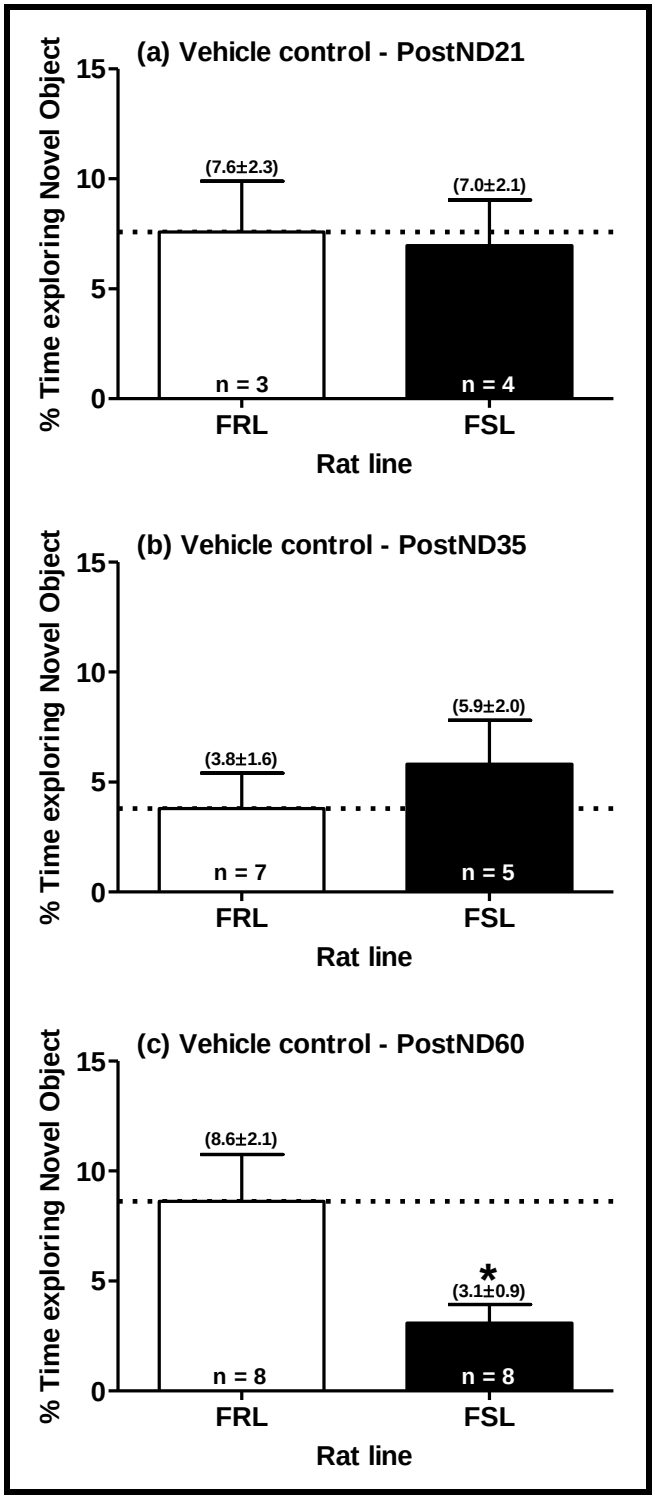



**Figure B-4: Percentage time spent exploring the novel object during the retention trial of the novel object recognition test by the FRL and FSL control groups on the different specified ages postnatal.**

**FRL and FSL control groups at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with the unpaired Student's t-test (two-tailed), with statistical significance taken as $p < 0.05$ (*).**

The data above suggests that, similar to that seen with depressive-like behaviours in the FST (*Figure B-2(c), page 96*), a significant level of cognitive impairment in the FSL control rats is only observed at PostND60 (*Figure B-4 (c), page 100*) and not at an earlier stage of life such as PostND21 (*Figure B-4(a), page 100*) or PostND35 (*Figure B-4(b), page 100*). This information needs to be taken into consideration when designing a study where the effect of drug treatment on cognition will be assessed at certain stages of development. These data then confirm that, as observed in the FST (*Figure B-2, page 96*), the FSL control rats only exhibit impaired memory consolidation at PostND60 and not at PostND21 or 35 compared to the FRL controls. This may indicate that memory consolidation impairment, is only observed in the post-pubertal (mature) FSL rat.

## B.2.4 The elevated plus maze

Finally, the anxiety-like behaviour of the FSL and FRL control rats were measured in the elevated plus maze (EPM) as the time spent in the open arm of the maze. These data are illustrated in *Figure B-5* (*page 102*), below.

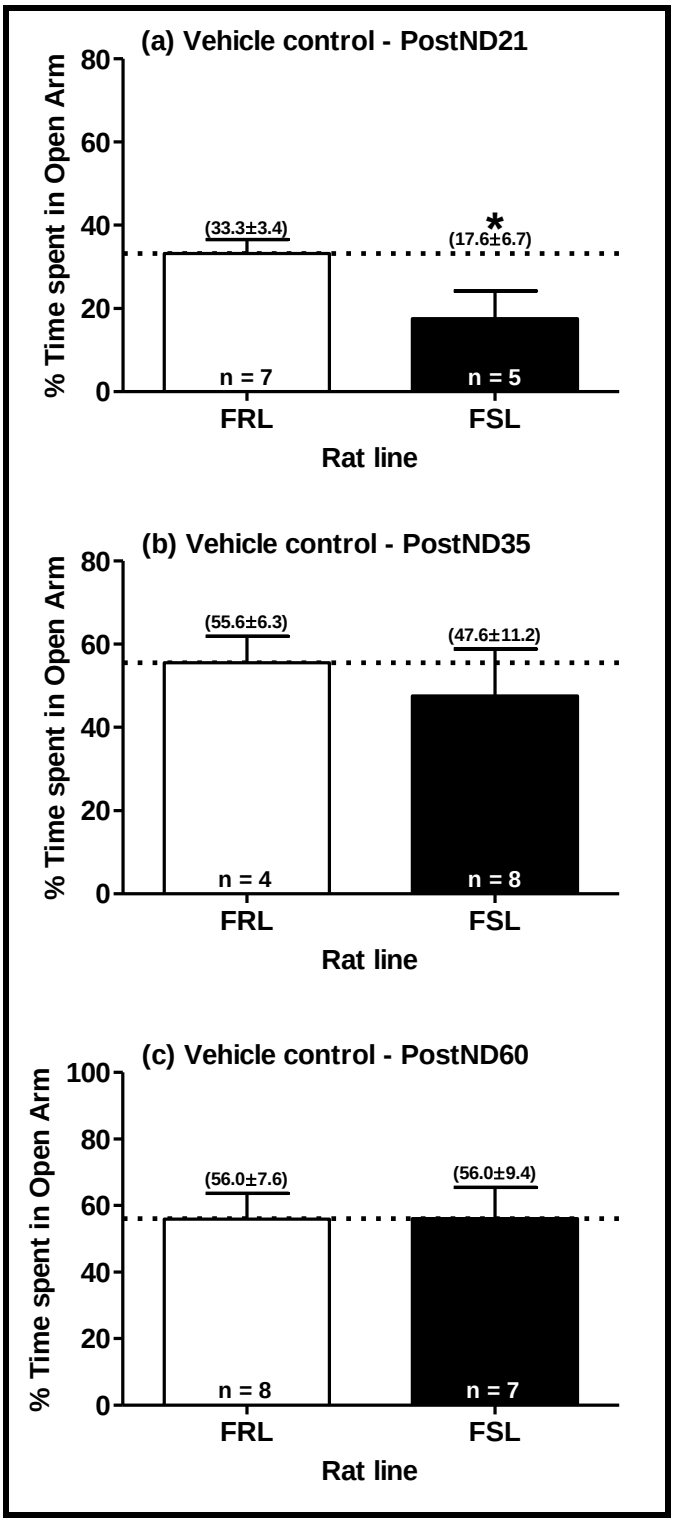



**Figure B-5: Percentage time spent in the open arm of the elevated plus maze test by the FRL and FSL control groups on the different specified ages postnatal.**
**FRL and FSL control groups at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with the unpaired Student's t-test (two-tailed), with statistical significance taken as $p < 0.05$ (*).**

It can be seen in (*Figure B-5(a), page 102*) that pre-pubertal FSL rats (i.e. PostND21) display significantly less anxiety, compared to their FRL controls. A similar trend was, however, not observed at PostND35 or PostND60 (*Figures B-5(b) and (c), page 102*). Increased anxiety levels are not a predominant feature of the FSL rats and has been reported to develop to levels displayed by adult FSL control rats (see discussion in *§ 4.2.3*). These findings were considered when designing the main study described in *Chapter 3*, where the effects of venlafaxine treatment on levels of anxiety would be assessed at certain stages of development. These data then confirm that the pre-pubertal FSL rats display significantly decreased anxiety-like behaviour, compared to their FRL control which is in accordance with previous studies indicating that pre-pubertal FSL rats develop the increased anxiety, as observed in adult FSL rats, in their post-pubertal years.

In conclusion, the data from the behavioural tests of the FSL and FRL control groups confirms the FSL rat as a validated animal model for depression as it exhibited several behaviours associated with depression, as previously documented (*§ A.1.1*), viz. increased immobility in the FST and reduced cognitive performance in the nORT. Importantly, increased levels of anxiety did not feature prominently at any age. The characteristic behavioural changes evident in the FST and nORT, however, were only observed in the adult rat (i.e. PostND60) and not in the pre-pubertal control groups (i.e. PostND21 or 35), suggesting a transient development of these characteristics as a factor of age (discussed in *§ 4.2.3*). Following chronic saline treatment, throughout pre-and postnatal development, the FSL rats exhibited increased immobility in the FST and decreased memory consolidation in the nORT, compared to their behavioural FRL control rats. Apart from the significant lower anxiety, displayed by the FSL control rats in comparison to their FRL controls, on PostND21, no other significant differences were observed in the two control rat lines on PostND35 or 60, suggesting that the FSL pre-pubertal rat, although not displaying depressive-like behaviour, may still be a valid model for paediatric depression as it showed a natural predisposition to such behaviour later in life.

## B.3 FRL versus FSL rats (venlafaxine treated groups)

After acquiring the baseline behavioural performances of the FRL and FSL control groups, the venlafaxine treated groups in the FST, EPM and nORT under conditions of chronic injection stress, it was now necessary to establish the effect of venlafaxine treatment, subjected to the same battery of tests, but following administration via four different dosing regimens (*§ 1.4*) under consideration for the main study. The following treatment regimens were investigated:

1. The behaviour and cognitive function of pre- and/or postnatal venlafaxine treated FSL and FRL rats, compared to that of FRL and FSL control rats, discussed in *§ B.2*.

Again, some of the data below have already been reported in table format in *§ 3.3.3*, while some have not, but are given below in order to enable a more comprehensive analysis of observations.

### B.3.1 The forced swim test

Firstly, the time spent immobile during the FST in FRL and FSL rats at the specified ages, and following the various venlafaxine treatment strategies, are illustrated below and compared to the respective control groups (*Figure B-6, page 105*), discussed earlier.

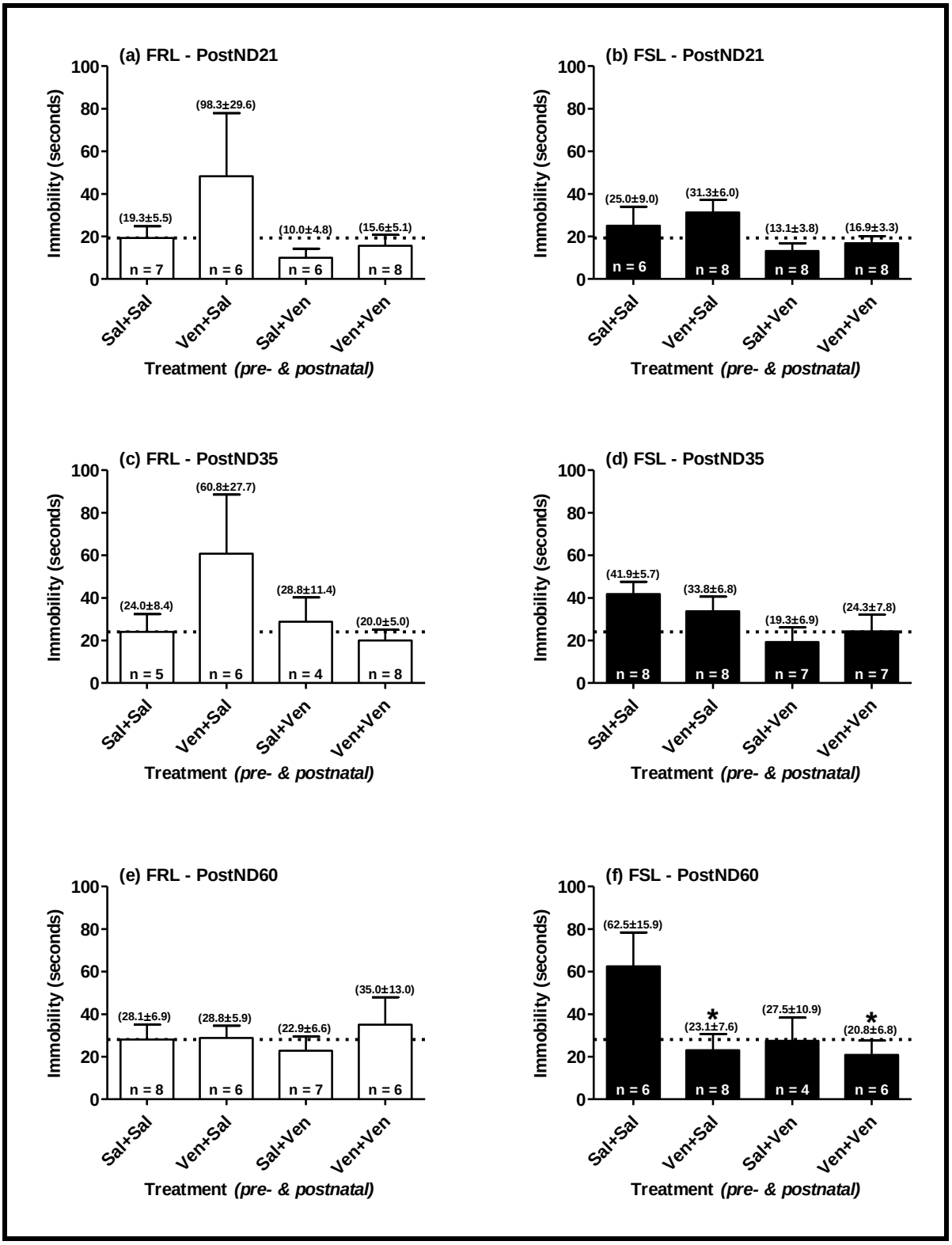



**Figure B-6: Time spent immobile during the forced swim test by the FRL and FSL rats on the specified ages postnatal, following venlafaxine treatment during the indicated early-life phases.**

**FRL and FSL venlafaxine treated groups during specified early-life phases at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with a one-way ANOVA and the Dunnett's post-test, with statistical significance taken as $p < 0.05$ (*).**

The data in *Figure B-6* suggest no effect on the depressive-like behaviour of FSL rats on PostND21 (*Figures B-6(b), page 105*) or PostND35 (*Figures B-6(d), page 105*), compared to their respective controls, regardless of the venlafaxine treatment regimen applied. However, venlafaxine treatment during the prenatal phase (Ven+Sal) and the pre- and postnatal phases (Ven+Ven), induced a significant decrease in depressive-like behaviour in FSL rats on PostND60 compared to the FSL control (*Figure B-6(f), page 104*) ($F(3,20)=3.441$; $p = 0.0364$). The lack of significant decrease in the depressive-like behaviour of the postnatal venlafaxine treated group (i.e. Sal+Ven) on PostND60, may be the result of insufficient statistical power (i.e. n=4; $p = 0.012$; Statistica v10). By further analysing the effect size $d = 0.90$, this suggest a practically significant effect and further supports the notion that the lack of statistical significance is due to a lack of statistical power.

Furthermore, regardless of the treatment regimen, venlafaxine did not affect the depressive-like behaviour of the FRL rats, compared to their controls in any of the three different age groups (*Figues B-6(a)*, *(c) and (e), page 105*).

In order to more specifically address whether venlafaxine was indeed modifying serotonergic and noradrenergic activity, we also scored swimming and climbing behaviour in the FST, an extension of the method that allows differentiation between serotonergic- and noradrenergic-related responses, respectively (Cryan et al., 2002). The data of the climbing and swimming behaviour in the venlafaxine treated FRL and FSL rats on PostND60 are illustrated in *Figure B-7* (*page 107*), below.

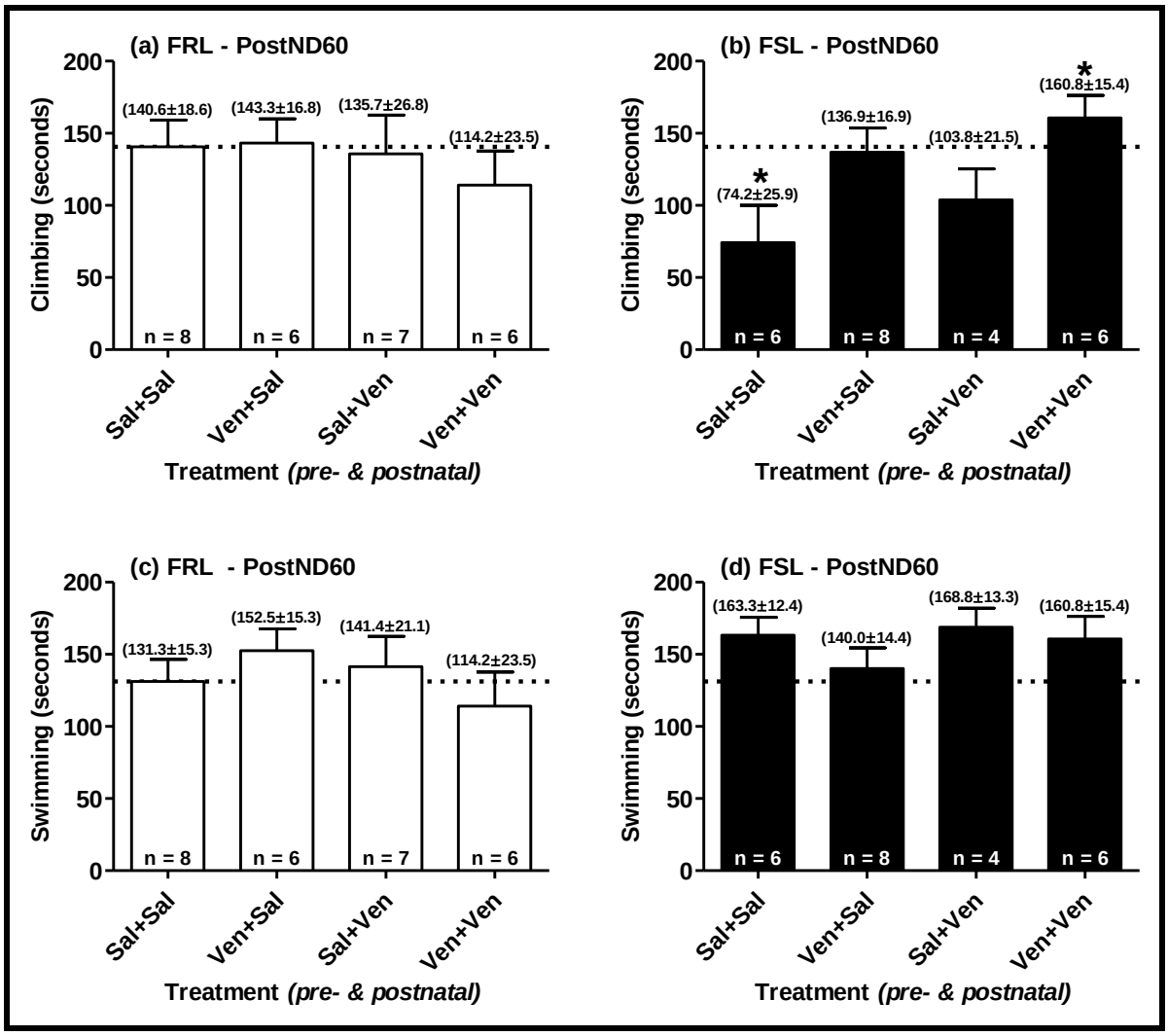



**Figure B-7: Time spent climbing and swimming during the forced swim test by the FRL and FSL rats on postnatal day 60, following venlafaxine treatment during the indicated early-life phases.**

**FRL and FSL venlafaxine treated groups during specified early-life phases at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with a one-way ANOVA and the Dunnett's post-test, with statistical significance taken as $p < 0.05$ (*).**

As will be discussed in *§ 4.2.4*, data from the current study indicated a significant increase in the climbing of the pre- and postnatal venlafaxine-treated FSL rats (i.e. Ven+Ven) on PostND60 (*Figure B-6(b) , page 105*) but not in the FRL group (*Figure B-6(a) , page 105*), while neither rat line demonstrated any effect on the time spent swimming (*Figure B-7(c) and (d)*) following the venlafaxine treatment. The climbing action displayed in the FST has been attributed to noradrenergic mechanisms, while the swimming action is related to serotonin mechanisms. Since venlafaxine is a dual 5-HT and NE reuptake inhibitor, the fact that venlafaxine increased climbing activity but not swimming, contradicts earlier findings (*§ 4.2.4*), as well as its purported mode

of action. Indeed, venlafaxine has a relative higher selectivity for the serotonin reuptake transporter compared to the norepinephrine reuptake transporter (see *§ 2.1.7.4*), and would therefore be expected to affect the swimming action to a greater extent than it does the climbing action. Clearly, these findings need to be confirmed. Considering these dubious findings, it was decided to exclude this aspect of the FST method from the main study until further confirmatory studies could be undertaken.

The above climbing and swimming results, obtained from the FST, suggest that although the serotonin pathways develop faster and reach maturity at an earlier stage than the noradrenergic system, administration of an SNRI (i.e. venlafaxine), seems to increase NE levels more than it does that of 5-HT. This increase in actions associated with NE, may represent a possible role for antidepressants targeting the NE system during early pre- and postnatal development.

## B.3.2 The Digiscan® animal activity monitor

Secondly, the locomotor activity, as measured in the Digiscan® animal activity monitor of FRL and FSL rats at the specified ages, and following the various venlafaxine treatment strategies, was studied. These data play an important role in the interpretation of the FST results (*§ B.2.2*) and are illustrated in *Figure B-8* (*page 109*), below.

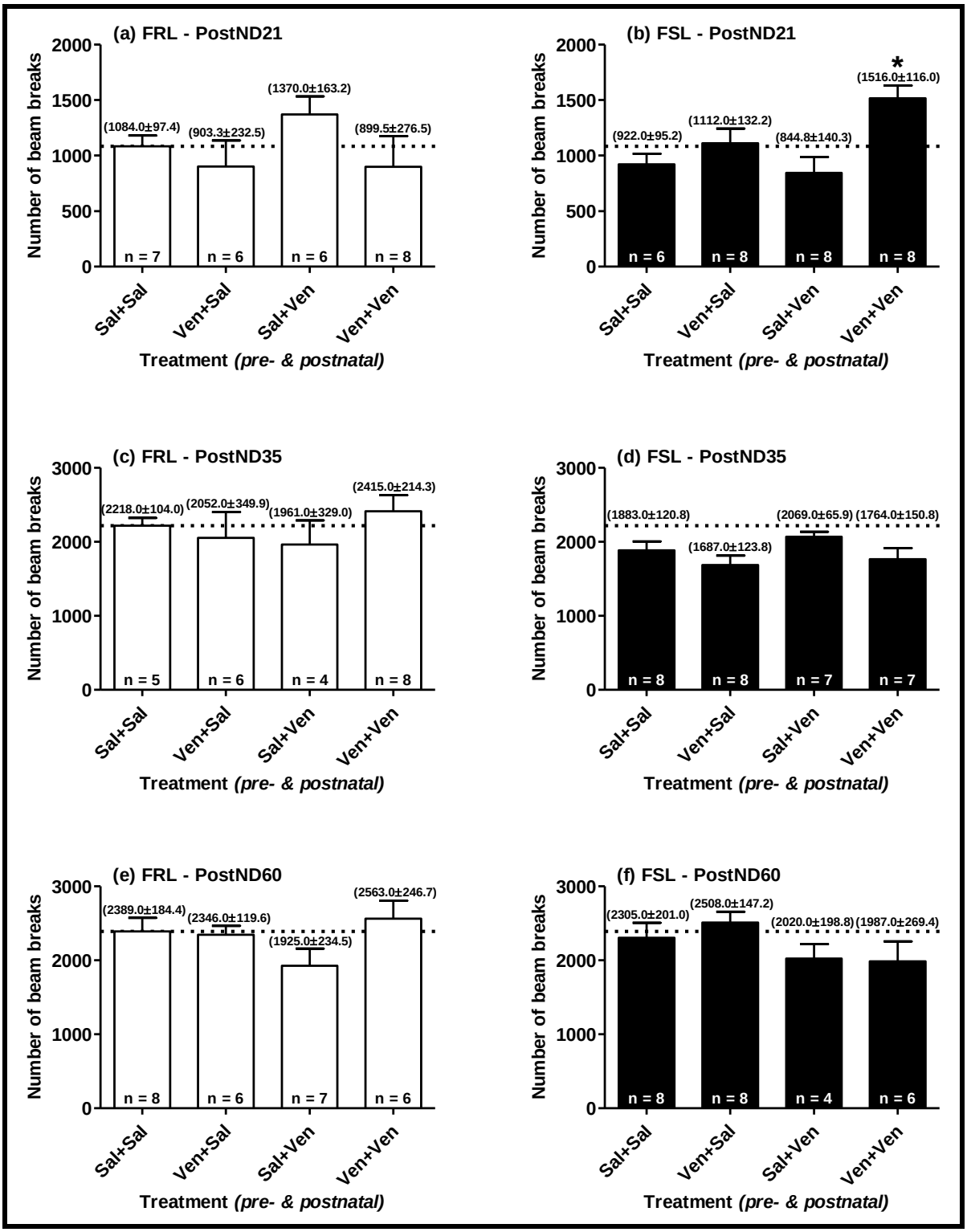



**Figure B-8: Locomotor activity in the Digiscan® animal activity monitor of the FRL and FSL rats on the specified ages postnatal, following venlafaxine treatment during the indicated early-life phases.**

**FRL and FSL venlafaxine treated groups during specified early-life phases at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with a one-way ANOVA and the Dunnett's post-test, with statistical significance taken as $p < 0.05$ (*).**

Firstly, venlafaxine treatment did not significantly affect the locomotor activity of the FSL rats, following prenatal (Ven+Sal) and postnatal (Sal+Ven) treatments, with the exception of a significant increase in the horizontal activity in the pre- and postnatal treated (Ven+Ven) rats on PostND21 (*Figure B-8(b), page 109*). This was, however, not observed in the FRL rats (*Figure B-8(a), page 109*). This increase was not observed in any of the venlafaxine treated FSL rats later in life i.e. PostND35 (*Figure B-8(d), page 109*) or PostND60 (*Figure B-8(f), page 109*) compared to their control groups, suggesting that the venlafaxine-induced increased locomotor activity in the stress-sensitive rats may be a transient effect (*§ 4.2.3*). However, the unaffected locomotor activity of the FSL rats on PostND60 supports the decrease in depressive-like behaviour observed in the PostND60 pre- and postnatal venlafaxine treated FSL rats (*Figure B-6(f), page 105*) as the increased time spent immobile cannot be associated with increased locomotor activity. The venlafaxine did not seem to alter the locomotor activity in any of the FRL rats, compared to their control groups (*Figures B-8(a), (c) and (e), page 109*).

## B.3.3The novel object recognition test

Thirdly, the general levels of memory consolidation (a measure of cognition) of the FRL and FSL control groups following the various venlafaxine treatment regimens, and at the three different stages of development, was undertaken. These data were generated by measuring the extent of memory consolidation in the retention trial of the novel object recognition test (nORT) in the different venlafaxine treated FRL and FSL rats, are illustrated below in *Figure B-9* (*page 111*).

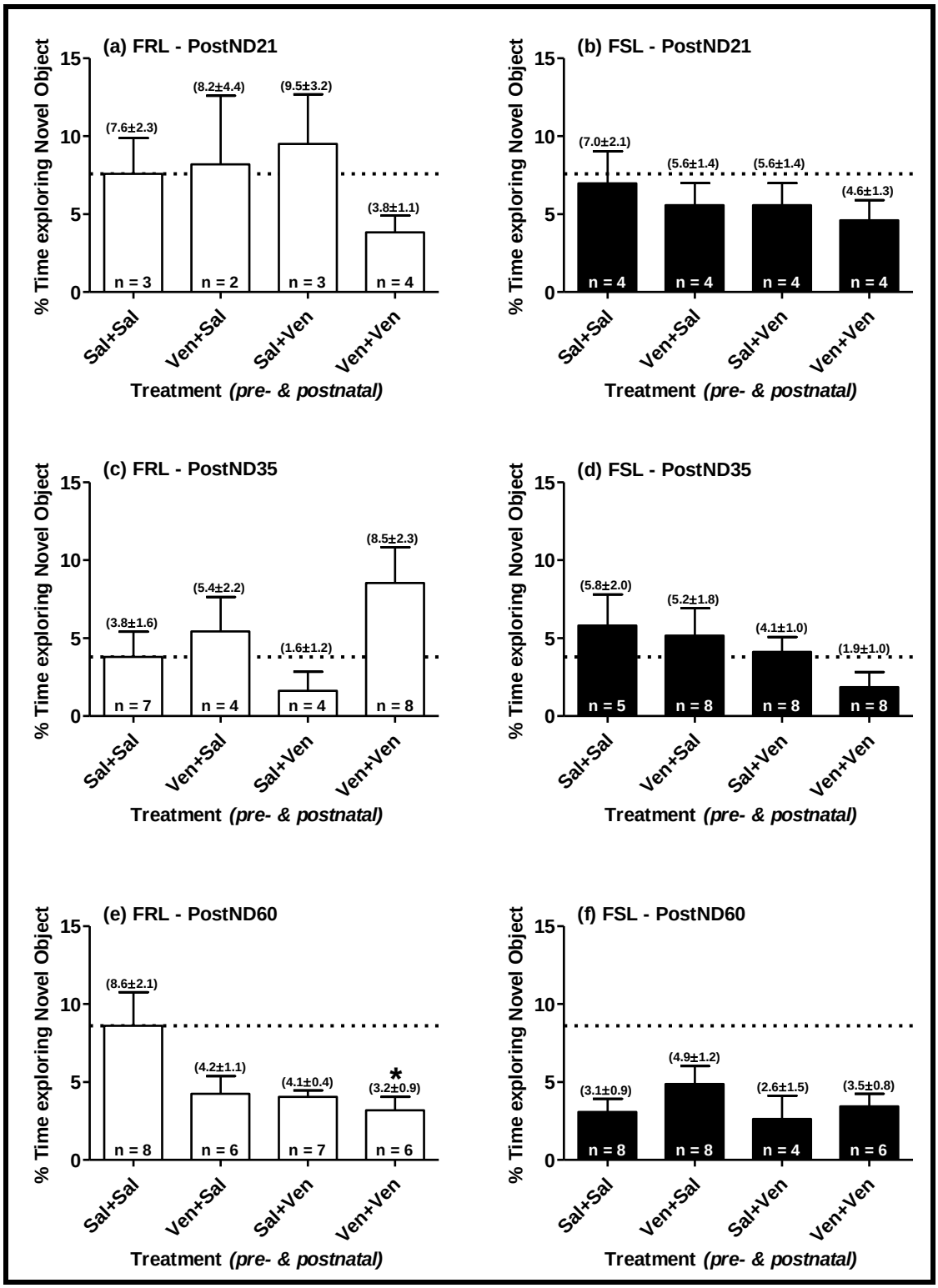



**Figure B-9: Percentage time spent exploring the novel object during the retention trial of the novel object recognition test by the FRL and FSL rats on the specified ages postnatal, following venlafaxine treatment during the indicated early-life phases.**
**FRL and FSL venlafaxine treated groups during specified early-life phases at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with a one-way ANOVA and the Dunnett's post-test, with statistical significance taken as $p < 0.05$ (*).**

Chronic venlafaxine treatment during pre- and postnatal development did not significantly affect memory consolidation of the FSL rats in the nORT at any age, compared to their controls (*Figures B-9(b), (d) and (f), page 111*). No significant alteration in the memory consolidation of FSL rats were observed on PostND60 (*Figure B-9(f), page 111*), whereas a significant reduction in the memory consolidation was observed in the pre- and postnatal venlafaxine treated (Ven+Ven) FRL rats at this age (*Figure B-9(e), page 111*). Although a similar trend was observed in the PostND60 FRL groups treated with venlafaxine only during the prenatal or postnatal phase, this did not reach statistical significance compared to the control group (*Figure B-9(e), page 111*). The venlafaxine-induced decrease in memory consolidation in FRL rats may suggest that the pharmacology of venlafaxine is different under neuropathological conditions than under normal conditions. If this would apply to the human condition (to be determined), it may also suggest that the neurodevelopmental consequences of early-life antidepressant administration on cognition may be less harmful in depressed patients than in normal individuals.

## B.3.4 The elevated plus maze

Finally, the anxiety-like behaviour of the FRL and FSL rats in the EPM, following chronic pre- and postnatal venlafaxine treatment, and at the three different stages of development, are illustrated in *Figure B-10* (*page 113*) below.

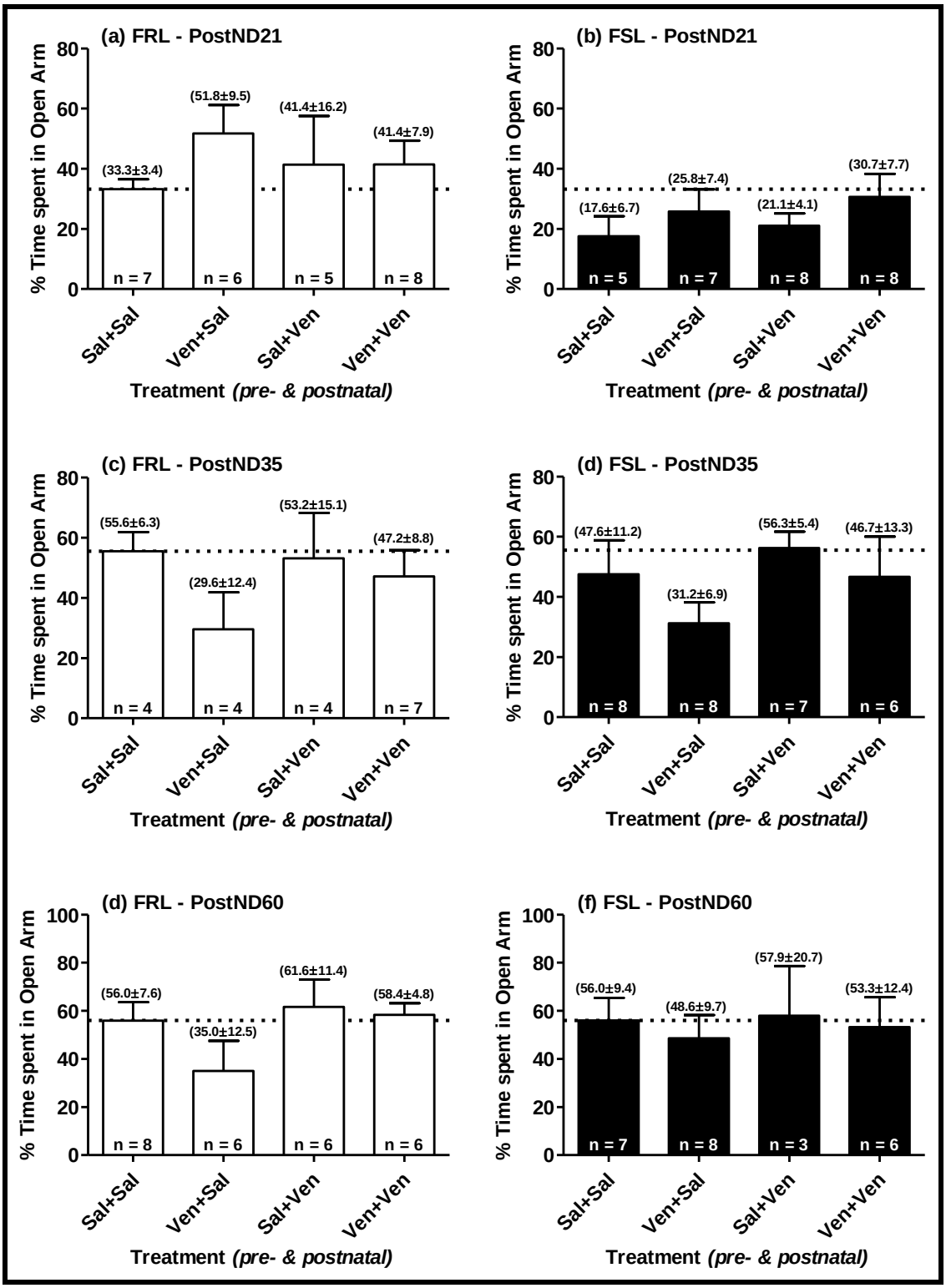



**Figure B-10: Percentage time spent in the open arm of the elevated plus maze test by the FRL and FSL rats on the specified ages postnatal, following venlafaxine treatment during the indicated early-life phases.**

**FRL and FSL venlafaxine treated groups during specified early-life phases at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with a one-way ANOVA and the Dunnett's post-test, with statistical significance taken as $p < 0.05$.**

As seen above, chronic venlafaxine treatment, during pre- and/or postnatal development, did not affect the anxiety-like behaviour of the FSL rats at any age (*Figures B-10(b), (d) and (f), page 112*), nor did it alter the anxiety-like behaviour of the FRL rats, compared to the control groups (*Figures B-10(a), (c) and (e), page 113*).

In addition to the time spent in the open arm of the EPM, the number of open arm entries acts as an expression of locomotor activity of the animals (see *§ 4.2.3*) and is illustrated in *Figure B-11* below.

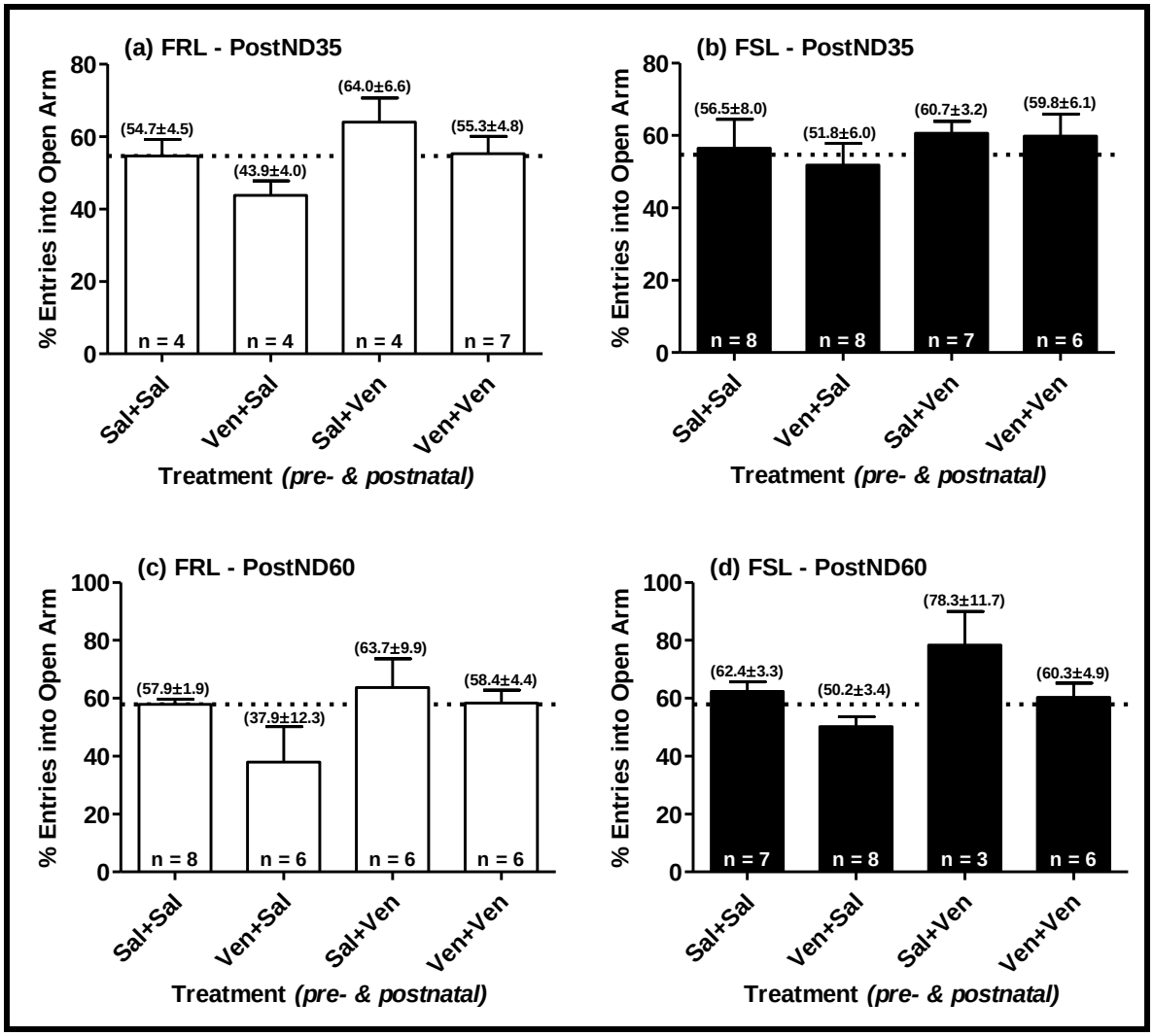



**Figure B-11: Percentage number of entries into the open arm of the elevated plus maze by the FRL and FSL rats on the specified ages postnatal, following venlafaxine treatment during the early-life phases.**

**FRL and FSL venlafaxine treated groups during specified early-life phases at postnatal day 21 (a), 35 (b) and 60 (c). Data are represented as averages ± S.E.M of duplicate observations from two independent and comparable experiments. Data were analysed with a one-way ANOVA and the Dunnett's post-test, with statistical significance taken as $p < 0.05$.**

As can be seen in *Figure B-11* (*page 114*), no significant difference in the number of open arm entries was observed between the venlafaxine treated FSL rats and their controls on PostND35 or 60. The comparable number of entries into the arms of the EPM, support the locomotor activity in the FSL rats as measured in the Digiscan® animal activity monitor (*Figure B-8(c-f), page 109*) and, therefore, supports the venlafaxine-induced decrease in depressive-like behaviour observed in the FSL rats on PostND60 (*Figure B-6(f), page 105*). The inclusion of the EPM was to investigate the anxiety-like behaviour of the FRL and FSL rats, following chronic venlafaxine treatment during early-life development, but as the control rats did not display any increased anxiety in comparison to their FRL controls, the EPM only indicated that the venlafaxine treatment did not increase or decrease anxiety compared to the FRL or FSL controls. In addition to the anxiety-like behaviour, the EPM supported the locomotor activity data by measuring the number of entries into the open arms and confirming the unaffected locomotor activity of the FSL and FRL rats on PostND35 and 60, following chronic venlafaxine treatment.

In conclusion, the FSL, compared to the FRL controls, displayed increased depressive-like behaviour in the FST as well as decreased memory consolidation in the nORT at PostND60 but not at PostND21 or 35. These data suggests that these characteristic behaviours of the FSL rat develops with age and only presents in early adulthood, corresponding with adult human patients who only develop MDD later in life even though they have a predisposed increased risk because of family members affected by the condition (see *§ 2.1.1.1*).

The venlafaxine treatment during early-life development significantly decreased the depressive-like behaviour of the stress-sensitive FSL rats at a later stage in life (i.e. PostND60), compared to the FSL control group of the same age. This decrease in depressive-like behaviour was, however not observed in the resistant FRL groups at PostND60, nor in any of the PostND21 and 35 venlafaxine treated FSL groups even though the same FST protocol was followed, suggesting that the hypotheses discussed in *§ 2.4* may have relevance and that with neurochemical investigation, these effects may be better explained.

In addition to affecting the depressive-like behaviour of the FSL rats later in life, the venlafaxine treatment during pre- and postnatal development did not alter the

anxiety-like behaviour or cognitive function of the FSL rats on PostND60, further suggesting that early-life treatment with a selective serotonin-norepinephrine reuptake inhibitor (SNRI) such as venlafaxine may be beneficial. A significant decrease in memory consolidation was, however, observed in the pre- and postnatal treated FRL group.

Finally, chronic venlafaxine treatment during early-life development did not seem to affect any of the parameters measured in any of the pre-pubertal FSL or FRL animals (i.e. PostND21 or 35), with the exception of the increased locomotor activity observed in the pre- and postnatal venlafaxine treated FSL rats on PostND21.

A complete summary and discussion of all the results in *Chapter 3* and *Addendum B* can be found in *§ 4.2*.

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Barnes, P.J., Karin, M., 1997. Nuclear factor-k β -a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med 336, 1066 -1071. Paivio, A., Jansen, B., Becker, L.J., 1975. Comparisons through the mind's eye. Cognition 37, 635-647.

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Strunk, W., White, E.B., 1979. The Elements of Style, third ed. Macmillan, New York, NY. Gurman, A.S., Kniskern, D.P., 1981. Family therapy outcome research: knowns and unknowns. In: Gurman, A.S., Kniskern, D.P. (Eds.), Handbook of Family Therapy. Brunner/Maazel, New York, NY, pp. 742-775.

*Order of references:*

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Maggi, C.A., Patacchini, R., Rovero, P., Giachetti, A., Meli, A., 1989b.

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# Congress contribution

In this addendum the study abstract that was presented at the congress of the South African Basic and Clinical Pharmacology Society (2011, Durban, South Africa) Young Scientist Category, is shown.

## Effect of Early-Life Exposure to the Serotonin-Norepinephrine Reuptake Inhibitor, Venlafaxine, on Behaviour in Adulthood in Stress-Sensitive Rats

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### Introduction

Depression during childhood and adolescence had been considered an uncommon condition, whereas more recent epidemiological studies suggest an alarming trend for a persistent escalation in the prevalence of depression in these age groups. Even though there is limited knowledge about the safety and long-term effects of treatment with antidepressants early in life on neurodevelopment and susceptibility to psychiatric disorders later in life, the number of prescriptions of these drugs for children and adolescents has increased significantly. The objective of the current study was to investigate the effects of early-life (pre-natal and post-natal) chronic treatment with the dual action serotonin-norepinephrine reuptake inhibitor, venlafaxine, in stress-sensitive rats on late-life measures of cognition, anxiety-like and depressive-like behaviour. In addition, the study also investigated which age was more associated with optimal behavioural changes later in life following the chronic administration of venlafaxine, viz. pre-natal versus early post-natal phase, or both.

### Methods

Stress-sensitive Flinder's Sensitive Line (FSL) rats and their controls, Flinder's Resistant Line (FRL) rats, were employed for the current study. Pregnant dams were injected subcutaneously for 14 days with 10 mg/kg venlafaxine or saline from pre-natal days 15 to 1. New-born pups were then injected subcutaneously with 3 mg/kg venlafaxine or saline for 14 days from postnatal days 3 to 17. Doses were determined from previous studies reported in the literature. Four rat treatment groups (n = 8/group) of both FSL and FRL rats received injections during pre-natal + post-natal ages as follows: saline + saline, venlafaxine + saline, saline + venlafaxine and venlafaxine + venlafaxine. Following the 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 postnatal day 21, 35 or 60 (separate treatment groups for each age group). 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**

Preliminary data suggest that none of the early-life treatment regimens influence behaviour or cognition in control FRL rats, as observed in the ORT, EPM or FST on post-natal days 21, 35 or 60. As expected, in stress-sensitive FSL rats following pre- and post-natal administration with saline control, depressive-like behaviour in the FST was significantly enhanced relative to corresponding FRL rat groups as observed at post-natal days 35 and 60, but not 21. Importantly, depressive-like behaviour as observed in FSL rats at post-natal day 60 was reversed following pre- and/or post-natal treatment with venlafaxine, relative to the corresponding FRL rat groups. Such reversal of depressive-like behaviour in FSL rats were not observed at post-natal days 21 or 35, suggesting a delayed response. Conversely, preliminary data from the ORT, LOCO or EPM did not reveal any significant differences between the various FSL treatment groups, including at post-natal day 60.

**Conclusions**

The current data therefore imply that early-life administration of venlafaxine to stress-sensitive (but not control) rats induce a delayed reversal of depressive-like behaviour, manifesting at post-natal days 35 and 60, but not earlier. Preliminary data do not support similar changes in anxiety-like behaviour or cognition.

# Abbreviations

| Numerals | |
|---|---|
| 5-HT | 5-Hydroxytryptophan (Serotonin) |
| 5-HT $x$ | 5-Hydroxytryptophan (Serotonin) $x$ -receptor subtype |
| α $x$ | Alpha $x$-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 $x$ | Dopamine $x$-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 |
| FST | Forced Swim Test |

## H

| | |
|---|---|
| H $x$ | Histamine $x$-receptor subtype |
| HIV | Human Immunodeficiency Virus |
| HPA-axis | Hypothalamic-pituitary-adrenal-axis |

## L

| | |
|---|---|
| *l*-NE | Norepinephrine |

## M

| | |
|---|---|
| MAO | Monoamine oxidase |

| | |
|---|---|
| MAOI | Monoamine oxidase inhibitor |
| MD | Major depression |
| MDD | Major depressive disorder |
| MDE | Major depressive episode |
| MHRA | Britain's Medicines and Healthcare products Regulatory Authority |
| MPFC | Medial prefrontal cortex |

## N

| | |
|---|---|
| nAChRs | Nicotinic acethylcholine receptors |
| NET | Norepinephrine transporter |
| nORT | Novel object recognition test |
| NE | Norepinephrine |

## P

| | |
|---|---|
| PDE 5 | Phosphodiesterase 5 |
| PFC | Prefrontal cortex |
| PostND $x$ | Postnatal day $x$ |
| PreND $x$ | Prenatal day $x$ |
| PVN | Paraventricular neucleus |

## R

| | |
|---|---|
| rCBF | Regional cerebral blood flow |
| REM | Rapid eye movement |
| RSA | Republic of South Africa |

| | |
|---|---|
| | **S** |
| Sal | Saline |
| s.c. | Subcutaneously |
| SERT | Serotonin transporter |
| SNRI | Serotonin-norepinephrine reuptake inhibitor |
| SSRI | Selective serotonin reuptake inhibitor |
| | **T** |
| TCA | Tricyclic antidepressant |
| | **U** |
| USA | United States of America |
| | **V** |
| Ven | Venlafaxine |
| | **W** |
| WHO | World Health Organization |

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