Early-life exposure to fluoxetine and/or exercise on bio-behavioural markers of depression in early adulthood in stress sensitive rats

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ABSTRACT

Juvenile depression is a major concern worldwide, with only the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and escitalopram approved for treatment. The effects of early-life exposure to SSRIs on neurodevelopment and subsequent lasting effects are not well understood. Exercise positively affects neuroplasticity, rendering exercise a potential augmentation strategy for drug therapy in juvenile depression. The current study investigated long-lasting behavioural and neurochemical effects of juvenile fluoxetine treatment and the potential role of exercise as treatment augmentation strategy in stress sensitive rats.

Male Flinders Sensitive Line (FSL) rats (n = 12 -16 per group), a well described, validated genetic animal model of depression, received either fluoxetine (5 mg/kg/day or 10 mg/kg/day subcutaneous) or vehicle control from postnatal day 21 (PostND21) to PostND34 (i.e. phase of pre-adolescence), together with simultaneous exposure to no, low or moderate intensity exercise (ethics approval no. NWU-00148-14-A5). Thereafter rats were housed normally and subjected to the open field test (OFT) and the forced swim test (FST) on PostND35 or PostND60 (early adulthood) to assess locomotor activity and depressive-like behaviour, respectively. Furthermore, euthanasia was applied to rats of the main study on PostND61, to assess hippocampal levels of brain-derived neurotrophic factor (BDNF), plasma levels of corticosterone, malondealdehyde (MDA) and superoxide dismutase (SOD).

On PostND35, 5 mg/kg/day fluoxetine, but not 10 mg/kg/day, significantly decreased immobility in the FST vs. vehicle control. This effect of 5 mg/kg fluoxetine was associated with enhanced climbing but no change in swimming behaviour in the FST, suggesting enhanced adrenergic but not serotonergic neurotransmission following early-life exposure. Neither low nor moderate intensity exercise altered immobility in the FST on PostND35 with also no changes in locomotor activity observed in the OFT. This effect of low intensity exercise was however associated with enhanced swimming behaviour, suggesting enhanced serotonergic neurotransmission following early-life exposure. The combination of fluoxetine 5 mg/kg/day and low intensity exercise significantly decreased immobility when compared to the sedentary plus vehicle control group on PostND35. This was associated with enhanced swimming behaviour. No changes were observed in locomotor activity.

On PostND60, following 26 days treatment-free housing, fluoxetine 5 mg/kg/day significantly decreased immobility vs. the vehicle plus sedentary control group, associated with increased climbing behaviour, again suggesting enhanced adrenergic neurotransmission. Locomotor activity, as measured in the OFT, was unaffected. Pre-pubertal low intensity exercise significantly decreased immobility in the FST on PostND60, also as a result of increased climbing behaviour. Fluoxetine plus exercise did not affect behaviour in the FST on PostND60, but did significantly decrease locomotor activity in the OFT when compared to the vehicle plus sedentary control group. Further, only SOD was significantly increased in

all treatment groups when compared to the vehicle plus sedentary group. BDNF was significantly decreased in the fluoxetine plus exercise group when compared to the vehicle plus exercise group. No differences were observed in lipid peroxidation (MDA) and plasma corticosterone levels in early adulthood.

The pre-adolescent period in rats therefore presents a window of opportunity during which neurodevelopment is highly plastic and can therefore be manipulated to result in detrimental or beneficial lasting effects. These lasting effects are dependent upon the neurodevelopmental age at the time of exposure, the dose of drug as well as the intensity of exercise. Furthermore the exercise intensity should be adapted according to age, in order to ensure training at the targeted intensity (% VO_2max). The current data further suggest, as a working hypothesis, that treatment of depression during pre-adolescence in humans should be tailored individually, in order to optimise early-life treatment and ensure lasting beneficial neurodevelopmental effects.

Keywords: Depression; Neurodevelopment; Fluoxetine; Exercise, Treadmill exercise, Flinders Sensitive Line rat; Depressive-like behaviour; Locomotor activity

OPSOMMING

Depressie onder jeugdiges is wêreldwyd ŉ groot probleem, met slegs die selektiewe serotonien heropname remmers (SSHRs) fluoksetien en esitalopram wat goedgekeur is vir behandeling. Die effek van blootstelling aan SSHRs gedurende vroeë lewe op neuro-ontwikkeling en die daaropvolgende volgehoue effekte is nog onduidelik. Oefening het ŉ positiewe effek op neuroplastisiteit, wat daartoe lei dat oefening ŉ potensiële versterkingstrategie tot geneesmiddel behandeling in kinders met depressie kan wees. Die huidige studie het dus die langtermyn gedrags- en neurochemiese effekte van die behandeling van jeugdiges met fluoksetien ondersoek, asook die potensiële rol van oefening as versterkingsbehandeling van stres-sensitiewe rotte.

Manlike Flinders Sensitiewe Lyn (FSL) rotte (n = 12 – 16 per groep), ŉ goed beskryfde, gevalideerde genetiese dieremodel van depressie, het óf fluoksetien (5 mg/kg/dag of 10 mg/kg/dag subkutaneus), óf draer-kontrole vanaf postnatale dag 21 (PostND21) tot PostND34 (d.i. die fase van pre-adolosensie) ontvang, met of sonder gelyktydige blootstelling aan geen, lae of matige intensiteit oefening (etiek goedkeuringsnr. NWU-00148-14-A5). Rotte is daarna normaal gehuisves en onderwerp aan die oopveldtoets (OVT) en geforseerde swemtoets (GST) op PostND35 of PostND60 (vroeë volwassenheid) om lokomotor-aktiwiteit en depressie-agtige gedrag, onderskeidelik, te assesseer. Verder is genadedood op rotte van die hoofstudie op PostND61 toegepas ten einde hippokampus-vlakke van breinafkomstige neurotrofiese faktor (BANF), plasma vlakke van kortikosteroon, malondialdehied (MDA) en superoksiesdismutase (SOD) te assesseer.

Op PostND35 het 5 mg/kg/dag fluoksetien, maar nie 10 mg/kg/dag nie, immobiliteit beduidend verlaag in die GST i.v.m. die draer-kontole. Hierdie effek van 5 mg/kg/dag was geassosieer met verhoogde klimgedrag, maar geen verandering in swemgedrag in die GST nie, aanduidend van verhoogde adrenergiese, maar nie serotonergiese aktiwiteit na vroeë-lewe blootstelling nie. Nie lae of matige intensiteit oefening het immobiliteit in die GST op PostND35 verander nie, met ook geen verandering in lokomotoraktiwiteit wat in die OVT waargeneem is nie. Hierdie effek van lae intensiteit oefening het swemgedrag beduidend verhoog, aanduidend van verhoogde serotonergiese neurotransmissie na vroeë-lewe blootstelling. Die kombinasie van fluoksetien 5 mg/kg/dag en lae intensiteit oefening het immobiliteit beduidend verlaag in vergelyking met die ongeoefende draer-kontrole groep op PostND35. Hierdie was geassosieer met verhoogde swemgedrag. Geen veranderinge in lokomotor aktiwiteit was waargeneem nie.

Op PostND60, na 26 dae van behandeling-vrye huisvesting, het fluoksetien 5 mg/kg/dag immobiliteit beduidend verlaag in vergelyking met die draer plus oefeningvrye kontolegroep, geassosieer met verhoogde klimgedrag, weereens ŉ aanduiding van verhoogde adrenergiese neurotransmissie. Lokomotoraktiwiteit, soos gemeet in die OVT, was ongeaffekteerd. Pre-pubertale lae intensiteit oefening

het ook immobiliteit beduidend op PostND60 in die GST verlaag, ook as gevolg van verhoogde klimgedrag. Fluoksetien plus oefening het nie gedrag in die GST op PostND60 beïnvloed nie, maar het wel lokomotor-aktiwiteit in die OVT beduidend verminder in vergelyking met die draer plus oefeningvrye kontrolegroep. Verder was slegs SOD beduidend verhoog in alle behandelingsgroepe in vergelyking met die draer plus oefeningvrye groep. BANF was beduidend verlaag in die fluoksetien plus oefening groep in vergelyking met die draer plus oefening groep. Geen verskille was waargeneem in lipied peroksidase- (MDA) en plasma-kortikosteroonvlakke in vroeë volwassenheid nie.

Die pre-adolosensie tydperk in rotte verteenwoordig daarom ŉ geleentheidsgleuf waartydens neuro-ontwikkeling hoogs vormbaar is en daarom gemanipuleer kan word om nadelige of voordelige blywende effekte te lewer. Hierdie blywende effekte is afhanklik van die neuro-ontwikkelingsouderdom ten tyde van die blootstelling, die dosis van die geneesmiddel en die intensiteit van die oefening. Verder behoort die intensiteit van die oefening aangepas te word volgens die ouderdom, ten einde te oefening teen die geteikende intensiteit (% VO_2maks) te verseker. Die huidige data suggereer verder, as werkshipotese, dat die behandeling van depressie gedurende pre-adolosensie in mense individueel aangepas moet word, ten einde vroeë-lewebehandeling te optimiseer en blywende voordelige neuro-ontwikkelingseffekte te verseker.

Keywords: Depressie; Neuro-ontwikkeling; Fluoksetien; Oefening, Trapmeule-oefening, Flinders Sensitiewe Lyn rot; Depressie-agtige gedrag; Lokomotoraktiwiteit

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"In order for man to succeed in life, God provided him with two means, education and physical activity. Not separately, one for the soul and the other for the body but for the two together. With these two means, men can attain perfection"

(Plato, fourth century BC).

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CHAPTER 1

1.1 Dissertation Approach and Layout

This dissertation is presented in article format. The essential data have been prepared for publication in an accredited scientific journal and is presented in Chapter 3. In addition, the literature review and conclusions concerning the study is taken up into separate chapters (Chapter 2 & Chapter 4). Furthermore, additional data was not included in the article, but is no less important in understanding the study as a whole and is therefore added in Addendum A. Addendum B contains the abstract of data presented at the annual congress of the South African Society of Basic and Clinical Pharmacology in conjunction with Toxicology SA, Wits University, Johannesburg (31 August – 02 September 2015). An outline follows with the aim of orienting the reader towards the essential elements of this document.

Problem statement, study objectives and study layout

- Chapter 1: Introduction

Literature Background

- Chapter 2 (literature review of study as a whole)
- Chapter 3 (article introduction)

Materials and methods

- Chapter 3 (article: materials and methods)
- Addendum A (additional materials and methods)

Results and discussion

- Chapter 3 (article: results and discussion)
- Addendum A (additional results and discussion)

Summary and conclusion

- Chapter 3 (article conclusion)

General discussion and conclusion

- Chapter 4: Summary and comprehensive discussion of the entire study synthesising the findings of the article and addendum A including recommendations for future studies

1.2 Research Problem

Major Depressive disorder (MDD) is one of the most challenging mental health problems of our time affecting mood, cognition and behaviour of an estimated 350 million people world-wide at any given point in time (Bylund, Reed 2007). Of concern is that it also involves juveniles, affecting 2.5% of pre-adolescent children, being the most common mental health disorder in this age group (Bylund, Reed 2007). Importantly, paediatric depression holds a fourfold enhanced risk of reoccurring in adulthood (Pine et al. 1998); in addition paediatric depression is a predictor of later childhood anxiety disorders and attention defecit hyperactivity disorder (ADHD), long-term depression, a high risk for disease persistence and is associated with enduring psychosocial difficulties and functional impairment in adulthood (Bufferd et al. 2012). Severe depression often leads to suicide (World Health Organization 2012), resulting in suicide being the fourth leading cause of death in pre-adolescent children (Hulvershorn, Cullen & Anand 2011). This highlights the need for safe and effective treatment strategies in children, even more so in light of the dramatic increase in the prescription rates for the selective serotonin reuptake inhibitors (SSRIs) group of antidepressants in this age group. Only two drugs have been shown to be effective in the treatment of major depression in juveniles and have hence been approved for this indication (see below). In addition, as the US Food and Drug Administration (FDA) issued a black box warning in 2004 due to an initial increased risk of suicidal ideation in this age group following the use of SSRIs (Klomp et al. 2014).

Fluoxetine is the only drug approved for the treatment of major depression in children 8 years and older, and escitalopram in adolescents 12 years and older (Soutullo, Figueroa-Quintana 2013). As with adulthood depression, relapse rates are high and remission rates low (Marais, Stein & Daniels 2009). That said, antidepressants remain the first line treatment in moderate and severe depression (Willner, Scheel-Krüger & Belzung 2013), whereas non-pharmacological interventions such as psychotherapy, life-style adjustments and support groups are used as augmentation strategy, or as monotherapy of mild depression. Fluoxetine and escitalopram are both SSRIs that increase serotonin concentration in the synapse by inhibiting the serotonin transporter (Kovačević, Skelin & Diksic 2010). Although the concentration is immediately increased the therapeutic effect of these drugs are only present after 3-4 weeks with remission only after 6-8 weeks. Thus we need new treatment options or augmentation strategies that are safe and effective in the treatment of juvenile depression.

The concern has been raised about the potential long-lasting consequences of early-life antidepressant treatment and how this external influence could affect neurodevelopment. Brain development is a complex process and adverse stimuli either from pharmacological or non-pharmacological interventions during this period could potentially alter the brain's functional integrity in adulthood (Gomes da Silva et al. 2012). Previous studies have demonstrated the significant impact of early-life treatment with antidepressants on neurodevelopment influencing neurobiological functioning in later

life often resulting in anxiety-like and depressive-like behaviour in adulthood (De Jong et al. 2006). Furthermore, juvenile social isolation severely affects rodent neurodevelopment, further highlighting the vulnerability of the developing brain. Social isolation can profoundly affect pre-frontal cortex functioning, such as disrupting synaptic plasticity and decrease dopamine and serotonin signalling (Baarendse et al. 2013), often leading to schizophrenia like symptoms with a strong depressive trait (Fone, Porkess 2008).

Therefore the current study investigated the effect of chronic, pre-pubertal administration of the antidepressant fluoxetine during a time of ongoing neurodevelopment on behaviour and neuromarkers of depression in early adulthood. In the current study we also investigated exercise as strategy in the treatment of juvenile depression. Exercise is generally regarded as a safe, even advisable approach, and some data suggest that it may be an effective augmentation strategy in children. Data from both pre-clinical and clinical studies suggest that exercise may support neurotransmission and neurotrophin availability (Marais, Stein & Daniels 2009, Marlatt, Lucassen & van Praag 2010, Bjørnebekk, Mathé & Brené 2010). Although exercise seems to be a favourable therapeutic option, no formal therapeutic strategy with physical activity has been developed for patients, and in particular juveniles, with major depression (Ströhle 2009). Finally, the potential augmentative effect of exercise on antidepressant response was also studied.

1.3 Study objectives

1.3.1 Primary Objective

The primary objective of this study was to assess the long-lasting effects of pre-adolescent exposure to vehicle control, fluoxetine alone, exercise alone or fluoxetine plus exercise on depressive-like behaviour and neuromarkers of depression, as displayed after wash-out into early-adulthood.

1.3.2 Secondary Objective

The secondary objectives of this study were done in order to achieve the primary aim:

- Confirm the FSL rat as an animal model of depression under our experimental conditions.
- Determine the maximal age-related exercise intensities at which pre-adolescent FSL rats can run on a treadmill during the pre-pubertal period, as expressed by VO_2 max.
- Calculate age-related low and moderate exercise intensities as percentages of the maximal intensity as well as determine the most effective exercise intensity to exert early anti-depressive-like effects
- Determine the most effective dose of fluoxetine and most effective exercise intensity that exerts early anti-depressant-like effects

- Establish whether the augmentation of fluoxetine with exercise exerts any early beneficial effects on behaviour.

1.4 Study Layout

Figure 1-1 depicts the study as a whole. The study comprised of five phases (1, 2a, 2b, 3, 4) and the main study, as outlined under study objectives (see §1.3) and explained in more detail below. The study employed pre-pubertal male FSL rats (see §2.6) as a translational animal model of depression.

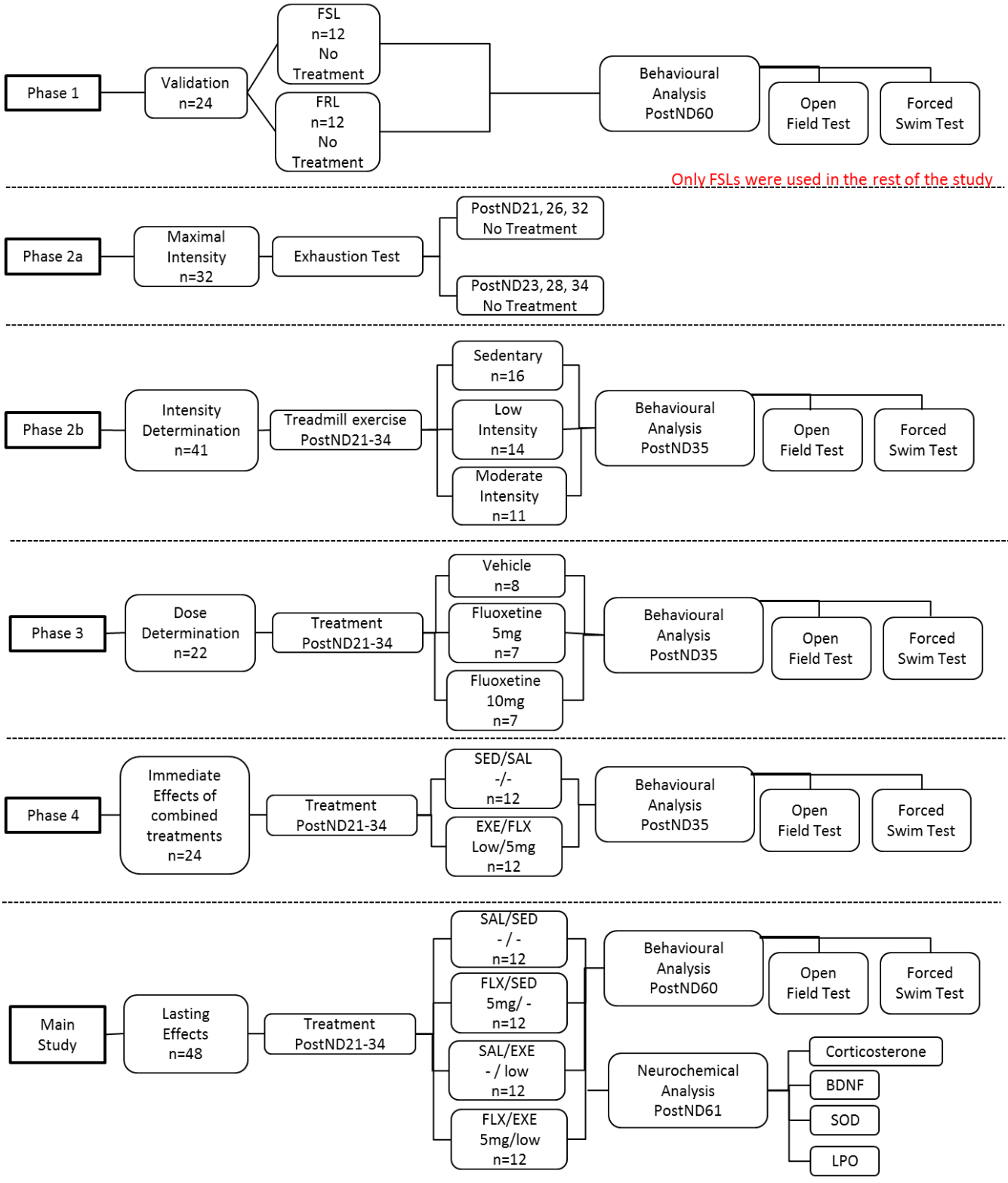

Figure 1-1: Study Layout of all phases (n=143) and Main Study (n=48)

Subjects were treated from PostND21 to PostND34 with either exercise, fluoxetine or a combination of the two as depicted in Figure 1-2. In order to decrease repetitiveness the layout as for the lifecycle of the rat will only be presented here. In the phases described hereafter, reference will only be made to the current figure. Some phases (2b and 3) had behavioural studies done on PostND35 whereas

other phases only did behaviour and/or neurochemical testing on PostND60. In the main study the same animals were used for both behavioural testing and neurochemical analyses.

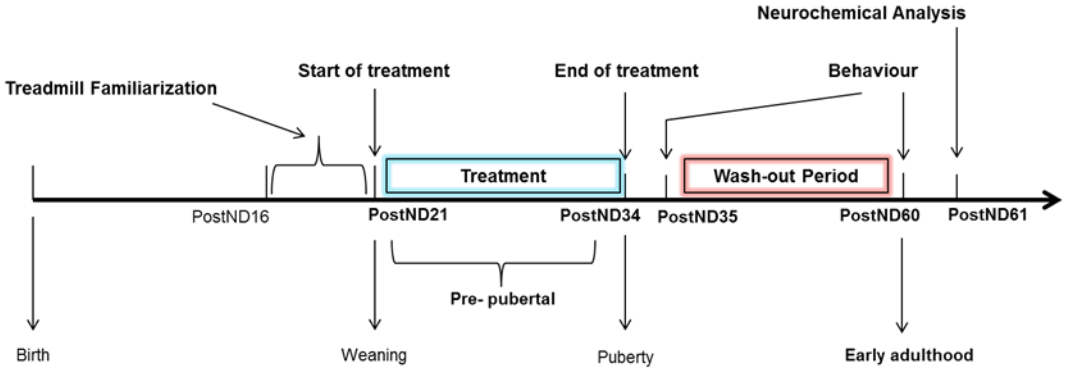

Figure 1-2: Study Layout as for the life-cycle of each rat. Only exercise studies Phase 2a&b) as well as the main study started with familiarization to the treadmill. However, this layout is not repeated in the texts that follows, but keep in mind that reference would be made to the current figure. Treatment included saline, sedentary, fluoxetine and exercise as indicated in each phase.

1.4.1 Phase 1: Validation of FSL as an animal model of depression

In order to validate the FSL as a translational genetic animal model of depression, male FSL (n=12) and FRL (n=12) rats were submitted to behavioural testing on PostND60. Animals were left in normal housing conditions from PostND21 (weaning) to PostND60. Behavioural analyses were performed i.e. the open field test (OFT) and the forced swim test (FST) in early adulthood. The OFT was done to assess locomotor activity, as well as determine any anxiety or lack thereof and the FST to determine depressive-like behaviour.

1.4.2 Phase 2a: Exhaustion Test

FSL rats (n = 32) were familiarised to the treadmill on PostND16-PostND20 as indicated in Figure 1-3 and described in §3.2.3.3. Between ages PostND21 to PostND34 FSL rats were divided into groups and submitted to exhaustion tests on different days as depicted in Figure 1-3 below, where after the data from these experimental procedures were used to calculate the different exercise intensities as a percentage of the maximal exercise intensity.

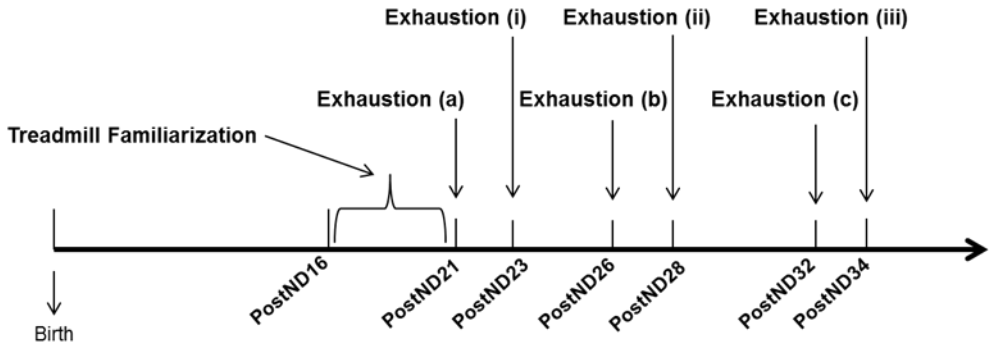

Figure 1-3: Layout of Exhaustion Test (n=32). One group of animals were subjected to an exhaustion test on days indicated as a, b and c whereas another group was subjected to exhaustion on days indicated as i, ii and iii.

1.4.3 Phase 2b: Effects of exercise intensities on depressive-like behaviour

In order to determine the most effective exercise intensity to exert lasting effects, FSL rats (n=41) were divided into 3 different groups (n ± 16) and submitted to different exercise intensities (sedentary, low or moderate) as determined in Pilot study IIa (§1.4.2) and described under exercise regimen in §3.2.3.4. On PostND16 rats were familiarized to the treadmill and submitted to daily exercise on PostND21 for 14 consecutive days and ended on PostND34 as indicated in Figure 1-2. On PostND35 animals underwent behavioural testing i.e. the open field test to determine any anxiety or lack thereof after chronic exercise. In order to determine the beneficial effects of forced exercise, the FST was also performed to determine depressive-like behaviour.

1.4.4 Phase 3: Immediate effects of fluoxetine on depressive-like behaviour

On PostND21 FSL rats were randomly assigned to groups and subcutaneously administered saline (vehicle), 5 mg/kg fluoxetine or 10 mg/kg fluoxetine daily from PostND21 to PostND34 (see Figure 1-2). After this treatment, PostND35 rats underwent testing in both the OFT and FST, in order to evaluate immediate effects of either dosage relative to the control. This was done in order to ensure that the fluoxetine was effective in the treatment of depressive-like behaviour in pre-adolescent FSL rats. The most effective dose was used in the main study.

1.4.5 Phase 4: Immediate effects of fluoxetine treatment combined with low intensity exercise

On PostND16 FSL rats were familiarised to the treadmill until PostND20. On PostND21 FSL rats were assigned to two groups and subcutaneously administered saline (vehicle), 5 mg/kg fluoxetine and/or subjected to daily low intensity exercise from PostND21 to PostND34 see (Figure 1-2). After this treatment, PostND35 rats underwent testing in both the OFT and FST, in order to evaluate immediate effects of augmentative therapy relative to the control.

1.4.6 Main Study

FSL male rats PostND21 described here under were randomly divided into a total of 4 different groups (n=12) (see Figure 1-1). Each group was submitted to either exercise (n=24) or no exercise (sedentary) (n=24). The exercise group (n=12) was assigned to a specific intensity as determined in pilot study IIb (§1.4.3). All of these exercised or sedentary (non-exercised) groups were administered either vehicle (n=24) or fluoxetine (n=24). Drug administration and exercise were done for 14 consecutive days, which is considered to be sub chronic.

Animals were familiarized to the treadmill and handling from PostND16 to PostND20 as seen in Figure 1-2 and described in greater detail in §3.2.3.1. Drug treatments and exercise regimen commenced on PostND21 and ended on PostND34, as suggested data from previous studies in our laboratory and other published studies indicated this as a neurodevelopmental phase correlating with human pre-adolescence (Steyn 2011).

The same animals were used for behavioural and neurochemical testing, to save animal numbers as well as costs. Animals were left to normal housing conditions from PostND35-PostND60 for a washout period and or forced inactivity of 26 days. On PostND60 (early adulthood), certain behavioural testing (OFT & FST– §3.2.4) was performed on all of the animals in each group to establish whether the interventions were effective in modulating locomotor, anxiety-like and depressive-like behaviour in early adulthood.

Neurochemical testing was done 24 hours after behavioural testing on the same animals. Animals were euthanized by decapitation and the hippocampus dissected (see §3.2.5) out. Brain tissue was snap frozen and stored at -80°C until neurochemical analysis.

1.5 Expected Results

Our working hypothesis is that exercise will augment fluoxetine treatment immediately after treatment as well as have positive lasting effects in a genetic animal model of depression. We then firstly postulate that pre-adolescent chronic administration of fluoxetine will reverse the depressive-like behaviour in FSL rats immediately following treatment (PostND35), and furthermore that this reversal of effects in FSL rats will have long-lasting effects into early adulthood (PostND60). Secondly, we postulate that low intensity exercise, but not moderate intensity exercise, will reduce the depressive-like behaviour in FSL rats. We thirdly postulate that exercise and antidepressants work synergistically, so that we expect to observe that the low intensity exercise regimens will augment the effects of fluoxetine, both immediately after treatment and continuing into early adulthood in FSL rats by reducing depressive-like behaviour as well as reducing neurochemical markers of depression.

1.6 Ethical Approval

All experiments conformed to the guidelines of the South African National Standards: The care and use of animals for scientific purposes (SANS 10386:2008) and were approved in accordance with the regulations set by the AnimCare animal research ethics committee (DoH reg. no. AREC-130913-015) of the North-West University, project ethics approval no. **NWU-00148-14-A5**.

CHAPTER 2

LITERATURE REVIEW

Childhood depression is a major concern worldwide and reportedly the most common mental health disorder in this age group (Bylund, Reed 2007). Still, the mere possibility that childhood depression exists, was once believed to be improbable, based on the naïve assumption that children cannot be prone to extremes in mood (Basu, Reddi 2009). Only by the turn of the previous millennium did epidemiological studies demonstrate that depression can in fact manifest in children (Weissman et al. 1999). This recognition prompted increased diagnosis of childhood depression, thereby spurring the initial perception of an increased prevalence of childhood depression (Bhatia, Bhatia 2007, Jane Costello, Erkanli & Angold 2006). In fact, there are still some who consider the prevalence to be on the rise (Weir, Zakama & Rao 2012). Nevertheless, the increase in the diagnosis of childhood depression and associated higher antidepressant prescription rate (Zito et al. 2002), frequent recurrence and the lack of neural recovery attributed to increased neuroplasticity resulting in increased vulnerability to both beneficial and diminishing effects of childhood antidepressant treatment (Branchi 2011, Andersen, Navalta 2004), have led researchers to investigate the long-term consequences of early life treatment.

2.1 Epidemiology

Major depressive disorder (MDD) affects 2-5% of the world population (i.e. roughly 350 million people world-wide at any point in time) (World Health Organization 2012, Bylund, Reed 2007) with a lifetime prevalence of 15% (Bylund, Reed 2007). In South Africa, the lifetime prevalence has been estimated to be 9.8% (Tomlinson et al. 2009). According to a study done by the Global burden of disease (GBD) in 2010, MDD was ranked the 2nd leading cause of years lived with disability (Kessler et al. 2015), having a major negative impact on global economy. The significant impact of MDD is due to its high prevalence, the severity of impairment of normal functioning and the life-threatening nature of the disease (Kessler et al. 2015).

MDD affects 4-8% of adolescents, up to 2.5% of pre-adolescents (Bylund, Reed 2007, Kessler, Avenevoli & Ries Merikangas 2001) and 0.3% of pre-schoolers (Kozisek, Middlemas & Bylund 2008). Recurrence of 40% after 2 years and 70% after 5 years has been demonstrated in children of school going age (6-12 years) (Luby et al. 2009). In a 2 year longitudinal study conducted by Luby and colleagues (2009) it was found that preschool depression, as with school-aged depression, has a high risk of recurrence as well as residual depressive symptoms even during recovery (Luby et al. 2009). Worldwide 20-25% of children aged 13-18 years will experience a depressive episode while others will experience subclinical symptoms of depression (Rubenstein et al. 2015, Bylund, Reed

2007). It is also during this adolescent phase when females will be more likely to develop depression than their male counterparts (Hankin et al. 1998). Interestingly, for the disease to reoccur in adulthood, paediatric depression predicts a fourfold increased risk (Pine et al. 1998, Rosso et al. 2005), pre-adolescent depression does not predict any significantly increased risk (Basu, Reddi 2009, Ryan 2005) and adolescent depression predicts a two- to fourfold enhanced risk (Bhatia, Bhatia 2007). In addition, paediatric depression is a predictor of later childhood anxiety disorders and ADHD (Luby et al. 2014, Bufferd et al. 2012), long-term depression (Ryan 2005, Pine et al. 1998), a high risk for disease persistence (Ryan 2005) and is associated with enduring psychosocial difficulties and functional impairment in adulthood (Weir, Zakama & Rao 2012, Pine et al. 1998).

Severe depression often leads to suicide, resulting in 1 million deaths every year (World Health Organization 2012) and being the fourth leading cause of death in pre-adolescents (Hulvershorn, Cullen & Anand 2011) and third leading cause of death among adolescents (Brown et al. 2013). This highlights the need for safe and effective treatment strategies in children, even more so in light of a dramatic increase in the prescription rate of SSRIs in this age group. In this regard, the prescription rate in children has been tapered following FDA warnings of suicidal ideation in this age group in 2004. Nevertheless, SSRIs still remain the most commonly prescribed class of drugs for treatment in children under the age of 18 (Karanges, McGregor 2011).

2.2 Signs, Symptoms and Psychopathology

MDD is a devastating disease, defined as a cluster of specific symptoms with associated impairment (Thapar et al. 2012) and characterised by the presence of anhedonia, characterised by a loss of pleasure or interest in pleasurable activities (Willner, Scheel-Krüger & Belzung 2013, Bylund, Reed 2007). Specific symptomatology and diagnostic criteria, as defined by the DSM-5, is described in par 2.3. Symptoms of depression include a persistent feeling of emptiness, hopelessness and worthlessness (Bylund, Reed 2007), often leading to significant psychosocial impairment, anxiety and even cognitive impairment which may include impaired attention and short-term memory (Bhatia, Bhatia 2007). Other common symptoms of depression include changes in sleep and appetite, problems with family and peers as well as substance abuse and/or suicidal behaviour (Bylund, Reed 2007, Ryan 2005).

MDD in pre-pubertal children is less common than MDD in adolescents or adults, and seems to differ from these disorders with respect to some causative, epidemiological, and prognostic features (Thapar et al. 2012). That being said, depression is a disorder with various symptoms trajectories that can emerge in childhood, only appear in adolescent years or appear in childhood and remit (Dekker et al. 2007). Children aged younger than 7 years may have difficulty in expressing their internal mood state and thus depression can manifest differently in the clinical setting (Bhatia, Bhatia 2007), such as vague somatic symptoms or even pain, other unexplained physical symptoms, eating disorders,

anxiety, refusal to attend school, decline in academic performance, substance misuse and/or behavioural problems (Thapar et al. 2012). Significant risk of self-harm and suicide is seen in adolescence. This complex presentation of symptoms complicates the diagnosis, challenges prescribers with treatment options and often results in persistence or recurrence when left untreated.

Various neurobiological changes are associated with depression in both children and adults. Despite the similarities in occurrence, clinical picture and longitudinal course of depression in children, adolescents and adults, there are notable differences in neurobiological correlates as well as in the response to treatment (Braw et al. 2006). Paediatric depression is associated with reduced rapid eye movement (REM) latency and REM density, hypercortisolaemia, increased inflammatory markers, reduced neurotrophic factors and alterations in frontolimbic and frontostriatal circuits (Rao 2013). However, children and adolescents do not show hypercortisolaemia as frequently as reported in adults (Braw et al. 2006). Children differ from adults in regards to basal cortisol secretion and corticotropin stimulation after corticotropin releasing hormone infusion (Braw et al. 2006).

The hippocampus and amygdala are limbic regions involved with regulation and memory of emotion and are reliably implicated in the aetiology and maintenance of depressive symptoms (Rosso et al. 2005). Studies have found significantly smaller left and right amygdalas in depressed children (Rosso et al. 2005). In a study conducted by Yap et al., (2008) it was found that boys (more so than girls) exposed to parental neglect had smaller amygdala volumes that correlated with more reports of depressive symptoms (Yap et al. 2008, Rosso et al. 2005). No changes in hippocampal volumes were found in children with depression or healthy control subjects (MacMillan et al. 2003), although some studies have found decreased hippocampal volumes, specifically left hippocampus volumes, in male adolescents (Hulvershorn, Cullen & Anand 2011). Adult hippocampal volumes have been found to be reduced in most but not all cases (Axelson et al. 1993). In this regard, atrophy of the hippocampus has been associated with lifetime duration of depressive episodes (Rosso et al. 2005), perhaps as a result of prolonged hypercortisolaemia (Sapolsky 2001). Of more interest is the fact that differences have been found in adults and adolescents, reflecting on-going neuroplastic changes and effects of depression on neural connectivity (Hulvershorn, Cullen & Anand 2011). Some researchers hypothesise that certain early neurobiological deficits predate psychopathology and has causal implications for the onset of depression, and that subsequent depressive episodes result in further neurotoxicity (McIntyre et al. 2013). This would emulate a vicious cycle of neuropathology and psychopathology.

2.3 Diagnosis

The symptoms for depression in young children and adults are broadly similar, although irritability is allowed as the core symptom in children, as opposed to depressed mood in adults (Thapar et al. 2012).

Furthermore, childhood depression often manifests in conjunction with other psychological diseases such as conduct and anxiety disorders (Rice 2014, Waszczuk et al. 2014).

According to the DSM-5 depression is diagnosed when 5 or more of the following symptoms have been present in the same 2 week period and represent a change from previous functioning and adhering to the following criteria (American Psychiatric Association 2013):

1. At least one of the symptoms should be either depressed mood for the greatest part of the day or loss of interest or pleasure (Essential Criteria). In children the mood might be irritable rather than sad.
2. Secondly the symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning.
3. The episode is not attributable to symptoms of another medical condition (Exclusion Criteria).
4. The occurrence of a major depressive episode (MDE) is not better explained by another psychotic disorder.
5. There has never been a manic episode or hypomanic episode.

Table 2-1: Diagnostic criteria for depression (American Psychiatric Association 2013)

Essential Criteria	Additional Criteria	Exclusion criteria
1. Depressed mood for the greatest part of the day 2. Loss of interest or pleasure in daily activities on most days	1. Significant weight loss or appetite changes 2. Altered sleep patterns 3. Frequent signs and symptoms of psychomotor agitation or retardation 4. Lack of energy or fatigue 5. Feeling of worthlessness 6. Difficulty in ability to think or concentrate 7. Continued contemplation of death	1. Effects of medication or drugs of abuse or manifestations of another disease

The main problems with depression in children include firstly the difficulty of diagnosis and secondly the lack of effective antidepressant treatment (Bylund, Reed 2007). More options are therefore needed to ensure the safety of children at risk of growing up to become depressed adults. Failure to appropriately treat mental disorders in juveniles can increase the likelihood of psychological problems later in life, but with limited data on the long-term effects of early-life treatment, prescribers are left with the daunting task of weighing estimated benefit and risk in deciding the best treatment.

2.4 Aetiology of depression

Depression is a complex disorder with no consensus on a simplistic, unifying neurobiological mechanism. Nevertheless, familial history (genetic make-up) seems to be the biggest risk factor, particularly in children (Nestler et al. 2002, Belmaker, Agam 2008). Over the past few decades researchers have developed several hypotheses of the neurobiological basis of depression in order to better understand its mechanism (Belmaker, Agam 2008), though fewer studies have been done to elucidate associations between genetics and neurobiological predisposition to depression. Even though data is relatively limited, evidence strongly suggest that interplay between genetics, the brain and the neuroendocrine system is also influenced by psychosocial risks and the environment (Pryce, Klaus 2013, Thapar et al. 2012).

The aetiology of depression in adults has been described extensively, predominantly focussing on the neurobiology underlying this complex disorder. However, a matter of debate is whether the aetiology of MDD in adults differs from that in childhood. This seems to be the case, as juvenile- and adult-onset depression shows different psychosocial risk profiles, with juvenile onset more strongly associated with family adversity, parental neglect, and problematic peer relationships (Thapar et al. 2012). It should be noted that, whereas the specific cause of depression in children may be different from that in adults, the overall neurobiology appears to be similar. Therefore current hypothesis of the neurobiological basis of depression, as typically associated with adult depression, would also be applicable to childhood depression, albeit with minor adjustments. The most important hypotheses for the neurobiological basis of depression that have been widely described include the following:

- Monoaminergic hypothesis
- HPA hyperactivity hypothesis
- Neuroplasticity hypothesis
- The immunological hypothesis
- Cholinergic super sensitivity hypothesis

Essentially, there is a strong relationship between the different postulated aetiologies of depression, and they are not mutually exclusive. That said, the high emergence of childhood depression is mostly related to genetic factors, and even more importantly all of the genes that have been associated with depression are directly or indirectly involved in the functioning of the immune system (Dantzer et al. 2008, Pryce, Klaus 2013). The role of the immune system in MDD is described in greater detail in par 2.4.5. However, it is important to note that the immune system influences the functioning of several other neurobiological systems and metabolic pathways such as the HPA-axis and serotonin production respectively, thus adding support to the idea of aetiologies influencing each other as well as overlapping mechanisms. Although depression is a disease of complex nature and overlapping

aetiologies, the various hypotheses for the aetiology of depression will each be described in greater detail below, starting off with genetic risk for depression and the influence of environment on genes followed by the hypotheses that aims to explain the neurobiological basis of depression.

2.4.1 Genetics and Gene-Environment hypothesis

Depression is a highly heritable disorder with the genetic risk estimated to be 40-70% (Nestler et al. 2002, Jacobson, Cryan 2007, Belmaker, Agam 2008). The non-genetic risk accounts for the remaining 30-60%, as the biggest risk factor for the development of depression in children and adolescents in addition to genetic susceptibility. These include environmental/psychosocial risk factors such as exposure to adverse events, stress, emotional trauma, drugs, viral infection and/or randomly dysfunctional processes during brain development (Nestler et al. 2002, Andersen 2003). However, genetic susceptibility and environmental stressors do not occur in isolation, but they rather interact with one another when they do co-occur. Therefore, depression has been proposed to result from interactions between a genetic predisposition and environmental influences (Lesch 2004).

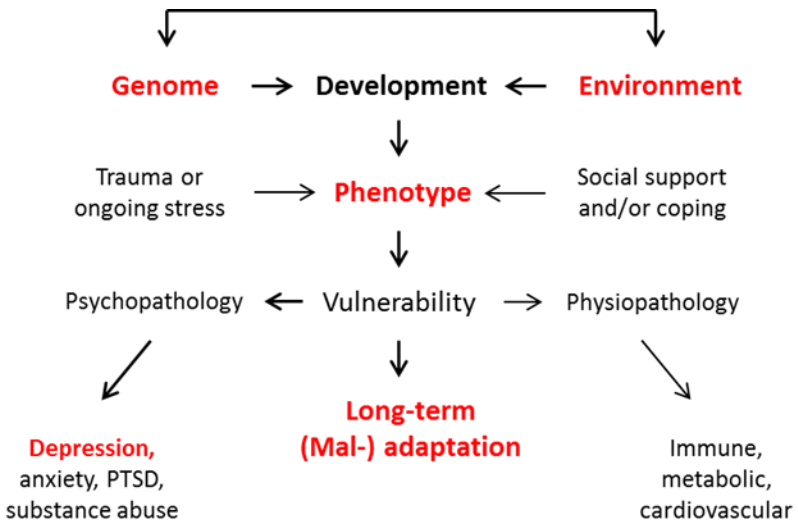

Figure 2-1: Conceptual model of the interaction between genetic predisposition and early environment leading to a vulnerable phenotype, as adapted from (Heim, Nemeroff 2001)

Development is determined by both the genetic makeup of the organism as well as environmental influences (see Figure 2-1). Specifically, both these factors can affect the maturation of brain circuits that are involved in affective functioning. The brain seems to be particularly sensitive to environmental disturbances during childhood period and consequently stressful events can affect cortical as well as limbic regions of the brain, including the frontal cortex, hippocampus and amygdala (Ansorge, Hen & Gingrich 2007). Given the role that these brain regions play in the regulation of various emotional and cognitive processes, as well as the effect of the environment on these regions, especially during development, has spurred the idea that depression may also in fact be viewed as a neurodevelopmental disorder (Hankin 2015). Consequently, adverse environmental

influences in a genetically susceptible individual will increase the likelihood of developing depression either in early life or during adulthood (Ansorge, Hen & Gingrich 2007, Caspi et al. 2003). Epigenetics may also be adversely affected by environmental stressors. Thus, early-life experiences or stressful situations can induce a long-lasting genetic 'scar', rendering a person more susceptible to depression later in life (Krishnan, Nestler 2008, Fone, Porkess 2008, Willner, Scheel-Krüger & Belzung 2013) see Figure 2-1. A clear example thereof can be seen with the social isolation model, an early-life intervention producing schizophrenia-like symptoms with a strong depressive link in rodents (Fone, Porkess 2008). That said, no single genetic or environmental factor can account for more than an estimated 5% of variance between depressed and normal patients, as confirmed in studies with twins that found that the vulnerability to depression is only partially linked to genetics, and that environmental factors also plays an important role (Andersen 2003).

Some epidemiological data also support the idea that some individuals are indeed genetically/neurobiologically more susceptible to develop MDD (Slavich, Monroe & Gotlib 2011). These patients tend to develop MDD after such stressful events that most other individuals might experience as minor forms of adversity (Slavich, Monroe & Gotlib 2011). Several factors may render an individual more vulnerable to stress. For example, Caspi and colleagues proposed that a functional polymorphism of the serotonin transporter (5HTT) gene may contribute, based on findings that individuals with a particular polymorphism of the 5HTT gene present more commonly with depressive symptoms and suicidality following stressful life events (Caspi et al. 2003). Baseline differences in serotonergic neurotransmission due to this gene can lead to early differences in emotional processing during exposure to stress, and hence an enhanced vulnerability to psychiatric disorders, including depression. It is also believed that the interaction between genes and the environment are particularly relevant if stressors are experienced during developmental phases where neuronal plasticity depends heavily on serotonin neurotransmission (Heim, Binder 2012). Similarly, others observed that single nucleotide polymorphisms in the gene encoding BDNF (Val66Met) are associated with increased occurrence of depression following stressful life events (Keers, Uher 2012). Even HPA-axis functioning has been shown to be sensitive to early adverse events, where early-life exposure to stressful events can lead to abnormalities in basal cortisol levels. These abnormalities in HPA-axis development and the influence thereof on neural circuitry can also be a possible reason for the development of depression in later life. Consequently it was suggested that genes (in particular corticotropin releasing hormone receptor 1 (CRHR1) gene) involved in the regulation of HPA-axis functioning may be involved in altered biological systems due to stressful experiences in early-life (Starr et al. 2014). Although the exact mechanism still has to be elucidated and several haplotypes for this gene exists, it is suggested that children carrying the CRHR1 risk haplotype are susceptible to a long-term increase in CRHR1 signalling. This could lead to a hyperactive stress hormone system following exposure to early trauma (Heim, Binder 2012).

Finally, it is important to note that environmental exposure can shape brain development either positively or negatively during certain critical periods of development (Andersen, Navalta 2011). For example; environmental enrichment, social support and antidepressants may modify the phenotype in a positive way, whereas chronic stress, drugs of abuse, stressful events and trauma can trigger depression, particularly during early development (Weir, Zakama & Rao 2012). That said, a negative outcome is often only expressed in the phenotype if the trigger occurs in the presence of a permissive genetic background (Lesch 2004, Heim et al. 2008). Interestingly, it has been suggested that the genes previously mentioned to be predictors of the vulnerability to depression in negative life events, may actually be beneficial in a positive environment, implying that gene-environment interactions may also reflect general sensitivity to environmental malleability (Heim, Binder 2012).

2.4.2 The monoamine hypothesis

The monoamine hypothesis postulates depression to be the consequence of impaired central monoaminergic neurotransmission, or in a simplistic view, the reduced availability of monoamine neurotransmitters in the central nervous system (Haase, Brown 2015, Berton, Nestler 2006, Nestler et al. 2002). Monoamines such as serotonin, noradrenalin and dopamine are distributed throughout the entire central nervous system, modulating many areas of emotion, thought and behaviour (Kuramochi, Nakamura 2009, Belmaker, Agam 2008).

Initial discoveries in the 1950s that drugs which enhance monoamine levels may alleviate depressive symptoms (Schildkraut 1967, Berton, Nestler 2006) and later findings that depressed patients have different monoamine profiles than normal patients (Maes et al. 2011, Ansorge, Hen & Gingrich 2007), served as the basis for the monoamine hypothesis in the aetiology of depression (Ressler, Nemeroff 1999). Norepinephrine (i.e. noradrenaline) has been implicated in the etiology of depression due to findings that reserpine, an anti-hypertensive drug that depletes catecholamines i.e. noradrenalin and to a lesser extent serotonin, causes depressive-like behaviour (Schildkraut 1967). Serotonin acts as the major modulatory neurotransmitter in the mammalian brain and plays a major role in neuroplasticity (Kepser, Homberg 2015). Serotonergic signalling pathways integrate not only basic physiological functions but also elementary tasks of sensory processing, cognition, emotion regulation and motor activity (Krishnan, Nestler 2008). Therefore it is not surprising that alterations in serotonin levels can lead to changes in mood, cognition, perception, sleep and appetite (Kepser, Homberg 2015). Although research into the function of dopamine in the pathophysiology of depression has been overshadowed by studies predominantly on norepinephrine and serotonin, it has long been known that dopaminergic neurons play a critical role in a wide variety of pleasurable experiences and reward. It is implicated in motivation, psychomotor speed, concentration and the ability to experience pleasure, whereas impairments of these functions are often seen in depression such as anhedonia (Dunlop, Nemeroff 2007). Due to the high incidence of depression in patients with

parkinsons disease, the role of dopamine in depression has been investigated extensively. It has also been found that responders to SSRIs have increased dopamine binding to D_2 receptors in the striatum as compared to non-responders (Dunlop, Nemeroff 2007).

Evidently, conditions disturbing monoamine networks in the brain significantly affects behaviour (Haase, Brown 2015). Numerous studies have found decreased monoamines in depressed patients as well as in post-mortem studies on the brains of depressed patients, adding further support to the monoamine hypothesis (Belmaker, Agam 2008). Heightened receptor expression, specifically of the $5HT_{2A}$ receptor in suicide victims (Schatzberg 2002) and depressed patients, suggests receptor upregulation due to reduced monoamine release. Nevertheless, findings of receptor expression is highly dependent on receptor type and brain region examined (Hamon, Blier 2013). These findings of monoamine deficiencies have driven antidepressant drug discovery, driving the development of drugs that facilitate serotonergic, noradrenergic and more recently dopaminergic neurotransmission in the brain (Hindmarch 2002, Booij et al. 2015).

The strong point of the monoamine hypothesis lies in its predictive power, since almost all antidepressants developed to inhibit the reuptake of serotonin and norepinephrine has been clinically effective (Belmaker, Agam 2008). It is of note that all antidepressants that have been developed to date influence the monoaminergic system in some way or another (Berton, Nestler 2006). The mechanism of action of these antidepressants depends predominantly on increasing the concentration of monoamine neurotransmitters in the synaptic cleft, increasing stimulation of the postsynaptic neuron and thereby alleviating symptoms of depression (Mahar et al. 2014, Belmaker, Agam 2008, Ansorge, Hen & Gingrich 2007). This increase in monoamines is primarily achieved by inhibition of the enzymes responsible for the transport of monoamines back into the neuron. Drugs inhibiting the norepinephrine and serotonin transporters have long been known to alleviate depressive symptoms and dopamine reuptake inhibitors developed for the use in Parkinson's have soon after the discovery of serotonin reuptake inhibitors also been found to be efficacious in the treatment of depression (Belmaker, Agam 2008).

However, a lack of universal efficacy of drugs that modulate monoaminergic neurotransmission (Krishnan, Nestler 2010) and the 2-3 week latency in response to treatment led researchers to recognise that the monoaminergic hypothesis may not fully explain all aspects of the aetiology of depression (Hindmarch 2002). Nevertheless, even novel treatment strategies that originally claimed to be completely unrelated to modulation of monoamines, was eventually found to influence monoamines in some or other way (Berton, Nestler 2006). Agomelatine, a melatonin receptor agonist for example, is not without effects on serotonin as it has predominant antagonistic effects on the $5HT_{2C}$ receptor (Papp et al. 2003), whereas tianeptine stimulates the re-uptake of serotonin (Mennini, Mocaer & Garattini 1987, Brink, Harvey & Brand 2006). Furthermore depletion of monoamines in healthy subjects fails to induce depression, whereas it causes a relapse in patients successfully treated

with SSRIs (Belmaker, Agam 2008, Maes et al. 2011), supporting the idea that there are other key neurochemical factors involved in the neuropathology of depression.

That said, the emphasis has not completely shifted away from the monoamines, but rather towards a syndrome of neural dysfunction (Groves 2007). Reductions in secondary messengers or reductions in the reaction of secondary messengers to stimulation by monoamine neurotransmission may impair the function of neurotransmitters even without affecting levels of monoamines (Belmaker, Agam 2008, Groves 2007). Although this indirectly involves the monoamine hypothesis, it could also explain the high rate of treatment failure. Focus has hence been redirected to the integral function of one monoamine in particular, namely serotonin, which has long been known to play a role in the aetiology of depression and has been investigated extensively (Limón-Morales et al. 2014). However, the renewed focus on serotonin has shifted the emphasis away from its primary function as a neurotransmitter to the recognition of the part it plays in vulnerability to develop depression. Compelling evidence suggest that the role of serotonin in neurodevelopmental processes (Whitaker-Azmitia 2001, Fakhoury 2015) and therefore the role of serotonin in also the development of depression. The putative association of serotonin with vulnerability to depression will be explained in greater detail in par. 2.7.

2.4.3 HPA-hyperactivity hypothesis

The HPA-axis plays a key role in biological coping mechanisms activated during psychological and biological stress, with its primary aim to maintain homeostasis (i.e. stability and health) (Heim et al. 2008). It is activated in both acute stressful situations and neuropsychological challenge, and also remains modulated, albeit differently, during chronic stress. The HPA-hyperactivity hypothesis of depression suggests a sustained activation of the HPA-axis as a product of an impaired negative feedback response (Pariante, Lightman 2008).

Prolonged increases of corticosteroids (cortisol in humans and corticosterone in rodents) can lead to hippocampal damage, as a result of reduced neuroplasticity (see neuroplasticity hypothesis below), involving neurogenesis and/or synaptogenesis (Pluchino et al. 2013). The hippocampus exerts a negative feedback response on cortisol secretion, so that damage to the hippocampus results in a reduction in the inhibitory effects of the hippocampus on the HPA-axis. Hippocampal dysfunction therefore could lead to extended increases in circulating corticosteroids, causing further harm to the hippocampus, thereby becoming a viscous cycle (Nestler et al. 2002, Belmaker, Agam 2008, Willner, Scheel-Krüger & Belzung 2013). Hyperactivity of the HPA-axis could also lead to immune activation and this inflammation can in turn stimulate the HPA-axis further, adding to the aforementioned viscous cycle (Pariante, Lightman 2008).

Hyperactivity of the HPA-axis is observed in the majority of depressed patients and in animal models of maternal separation (Berton, Nestler 2006, Belmaker, Agam 2008, Ansorge, Hen & Gingrich

2007). It also appears as if the adolescent brain may be more sensitive to stress than the adult brain (Eiland, Romeo 2013), suggesting that any direct effect of stressors on brain plasticity may be exacerbated in the adolescent brain. Nevertheless it has been suggested that the HPA-axis hyperactivity might not be the consequence of depression, but rather the manifestation of persistent neurobiological abnormalities that predispose depression (Pariante, Lightman 2008).

2.4.4 Neuroplasticity hypothesis

The neuroplasticity hypothesis postulates depression to be the result of impaired neuronal plasticity, including synaptic and structural plasticity. This proposes that, in the presence of aversive stimuli or environmental stressors, the lack or inability to appropriately adapt eventually result in the development of depression as depicted in Figure 2-2 (Schmidt, Duman 2007, Dwivedi 2009). This is supported by overwhelming evidence of altered synaptic and structural plasticity found post mortem in depressed patients, and in particular in the hippocampus of suicidal patients (Mahar et al. 2014) and healthy rodents exposed to stress (Bjørnebekk, Mathé & Brené 2010).

The hippocampus, a brain structure that is known to control emotion, learning and memory, is implicated as a key region in depression (Groves 2007). In this regard the hippocampus is a highly stress-sensitive region, especially during early-life development (Duman, Malberg & Thome 1999). Furthermore, during adulthood chronic stress leads to atrophy of the hippocampus (as discussed in par. 2.4.3) (Groves 2007, Hindmarch 2002).

Neurotrophic factors play an important role in hippocampal neurogenesis by means of cellular proliferation, migration and maintenance in the developing central nervous system. Neurotrophins are in part responsible for plasticity and survival of neurons during development and consequently decreases in neurotrophins could mediate structural damage and reduced neurogenesis in the hippocampus (Berton, Nestler 2006). Not only do neurotrophins play an integral role during development but are also required in the adult central nervous system for maintenance of neuronal functions, structural integrity of neurons and neurogenesis (Dwivedi 2009). Alterations in neurotrophin levels have been shown to result in structural abnormalities and reduced neuroplasticity, thereby impairing an individual's ability to adapt to stressful situations (Dwivedi 2009).

Of all the neurotrophins BDNF, has received the most attention as it is also implicated in modulating antidepressant-related behaviours (Mahar et al. 2014). For example, BDNF infusion exerts anti-depressive effects (Kozisek, Middlemas & Bylund 2008), whereas stress reduces BDNF expression. Early-life stressors such as social isolation decrease BDNF expression, correlating with depressive-like behaviour in adulthood. Administration of antidepressants, on the other hand, increases neurogenesis, synaptogenesis and neural maturation, again correlated with enhanced BDNF expression (Duman, Malberg & Thome 1999, Kozisek, Middlemas & Bylund 2008).

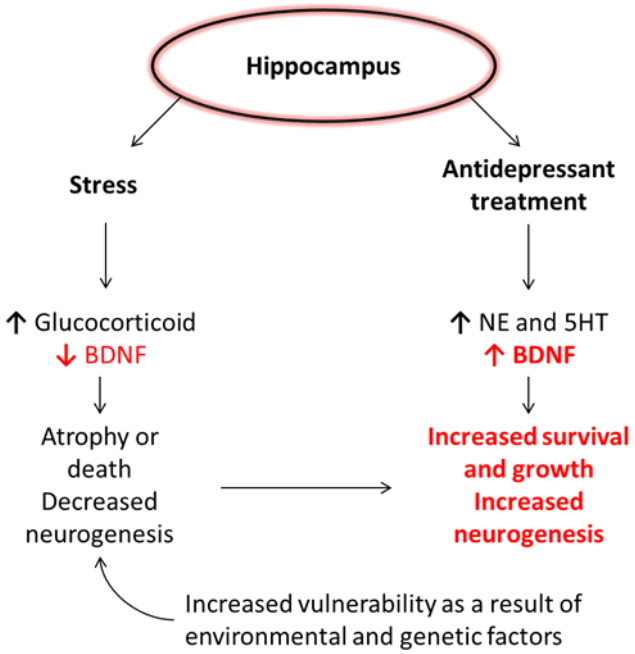

Figure 2-2: Generalised diagrammatic representation of the effects of stress and glucocorticoids on the hippocampus mainly via decreased expression of BDNF and how it is opposed by antidepressant treatment. Individual vulnerability could also occur as a result of genetic and environmental factors. Adapted from (Duman, Malberg & Thome 1999)

2.4.5 Immunological hypothesis

The immunological hypothesis of MDD postulates that depression is a psychoneuroimmunological disorder and has relevance particularly since activation of the immune system has been shown to be involved in stress and depression (Hindmarch 2002). Pre-clinical findings have consistently involved the immune system in depression, more specifically with an immunological animal model of depression. Rodents injected with the pro-inflammatory cytokine, lipopolysaccharide (LPS), displays systemic inflammation accompanied with depressive-like behaviour (Dantzer et al. 2011). Compelling evidence also suggests the role of cytokines in humans with MDD, being the most commonly measured immune biomarker in depression (Lopresti et al. 2014). Higher concentrations of tumour necrosis factor (TNF-α), interleukin (IL)-6, and IL-1 are commonly found in depressive patients (Lopresti et al. 2014). Furthermore chronic treatment with antidepressants such as TCAs and SSRIs has been found to reduce immune activation and cytokine synthesis (Schiepers, Wichers & Maes 2005). Inhibitors of TNF-α used for the treatment of rheumatoid arthritis have also been associated with alleviating depressive symptoms that accompany this disease (Schiepers, Wichers & Maes 2005).

Activation of the immune system influences many other systems and metabolic pathways in the human body through several mechanisms (Hindmarch 2002, Duman, Malberg & Thome 1999). One such pathway that has received a great deal of attention is the kynurenine pathway (Figure 2-3) being

the primary metabolic pathway of tryptophan, which acts as the main amino acid precursor of serotonin. Enhanced oxidation of tryptophan by Indoleamine 2,3 dioxygenase (IDO) to form kynurenine has been linked with several neuropsychiatric diseases, including MDD (Gałecki et al. 2009), schizophrenia and bipolar disorder (Maes 2011, Möller et al. 2013).

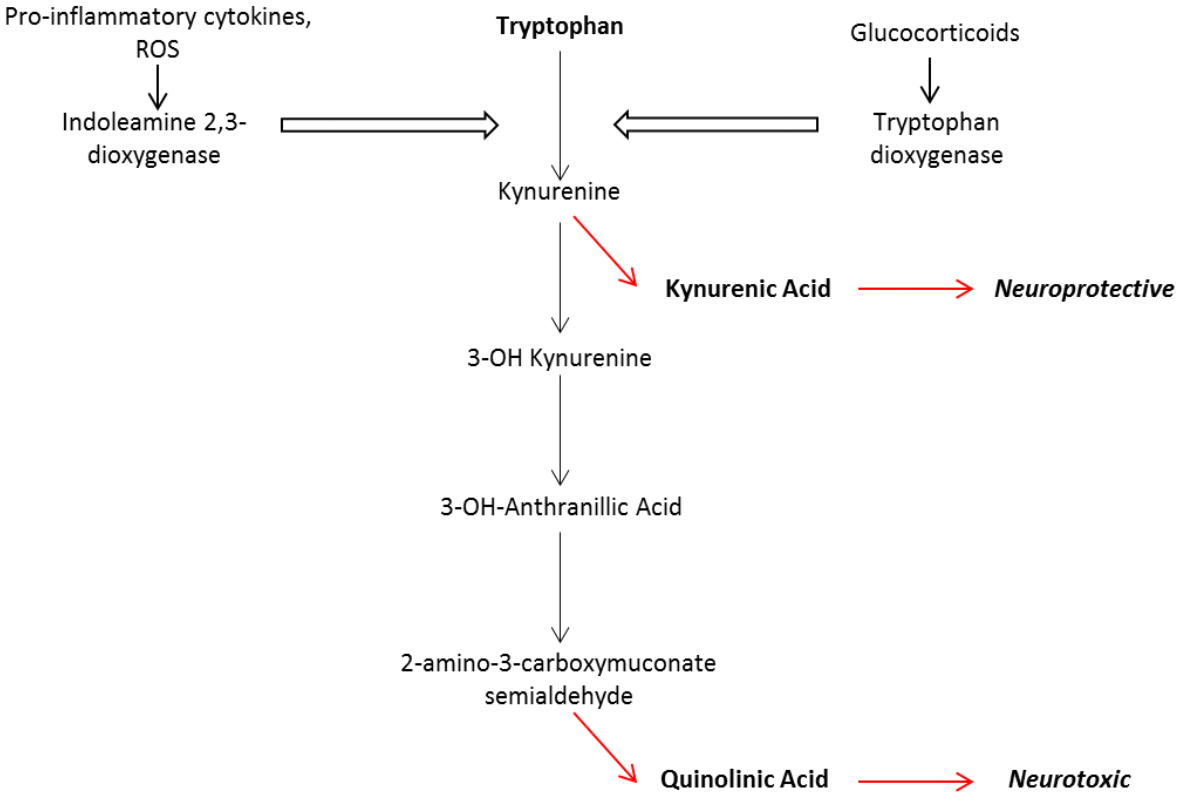

Figure 2-3: Effect of inflammatory markers on the kynurenine pathway {Adapted from (Maes 2011)

Cytokines in particular activates IDO (see Figure 2-3), inducing the break-down of tryptophan to kynurenine (Dantzer et al. 2008), producing less of down-stream kynurenic acid (neuroprotective) (Lopresti et al. 2014). This is believed to contribute to lower levels of serotonin, which is also in line with the monoamine hypothesis of depression (Dantzer et al. 2008). Decreased tryptophan and increased kynurenine in the peripheral blood of patients treated with IFN-α are associated with the development of depression (Bonaccorso et al. 2002). Kynurenine itself is neurobiologically inactive, but can be metabolised to different active metabolites, depending on the cell type. In microglia kynurenine is degraded to 3-hydroxykynurenine and quinolinic acid, both of which are neurotoxic due to their ability to generate oxidative radicals and to act as agonists at the N-methyl-D-aspartate (NMDA) receptor (Dantzer et al. 2011). The NMDA receptor is the primary binding site for glutamate. Glutamate is the major excitatory neurotransmitter in the central nervous system and synaptic neurotransmission that largely regulates mood and cognition (Sanacora, Treccani & Popoli 2012) as well as regulating neuroplasticity, learning and memory (Mathews, Henter & Zarate Jr 2012). Several new drugs exert their effects through the NMDA receptor. Ketamine for example is a

non-competitive NMDA receptor antagonist that shows rapid antidepressant effects (Laje et al. 2012) even in treatment resistant depression with effects after a single dose sustained for 7 days (Wang et al. 2015). However the detailed mechanism of glutamate and the NMDA receptor is beyond the scope of this dissertation.

Systemic inflammatory responses are frequently accompanied by induction of oxidative and nitrosative stress (O&NS) pathways (Maes 2011). These pathways produce radical oxygen species (ROS) and radical nitrogen species (RNS), with particularly ROS being implicated in the pathogenesis of various neuropsychiatric disorders, including major depression (Khanzode et al. 2003). These radicals react with proteins, fatty acids, and DNA, and cause damage to the structural cell wall and the mitochondria, eventually resulting in apoptosis and cell death (Maes 2011). ROS increases lipid peroxidation and hence oxidative stress, but also, via feedback mechanisms, induces protective antioxidant systems (Bilici et al. 2001). The brain contains large amounts of unsaturated fatty acids, catecholamines and monoamines, which are the target molecules for lipid peroxidation (Khanzode et al. 2003). Malondialdehyde (MDA), a marker of lipid peroxidation, concentrations are increased almost without exception in depressed patients, as compared to healthy control groups (Khanzode et al. 2003). The rise in MDA levels indicates membrane damage due to lipid peroxidation and increased oxidative stress in depressive patients (Khanzode et al. 2003). The brain has limited anti-oxidative defences, which makes it particularly vulnerable against such an attack. Defective antioxidant defences coupled with increased monoamine catabolism in depression may result in increased production of reactive oxygen species. It also appears that the hippocampus, one of the most notably affected brain regions in MDD, is the most susceptible to oxidative stress (Patki et al. 2013). Disturbances in the antioxidant super oxide dismutase (SOD) activity are generally found in depressed populations. However, findings are inconsistent regarding the direction of this disturbance, where some studies demonstrated a rise in serum SOD levels of MDD patients as compared to healthy volunteers (Bilici et al. 2001, Rybka et al. 2013, Herken et al. 2007, Stefanescu, Ciobica 2012), whereas others found a decrease in serum SOD in some patients (Bilici et al. 2001, Kodydková et al. 2009), as well as lower SOD in the frontal cortex, hippocampus and striatum after chronic stress (Ahmad et al. 2010).

Finally, further support for a redox hypothesis for MDD is that the antidepressant fluoxetine has been shown to restore the depletion of antioxidants and prevent oxidative damage (Kotan et al. 2011), while antioxidants like N-acetyl cysteine (NAC) have antidepressant-like properties in rats (Ferreira et al. 2008), as well as has antidepressant properties in bipolar (Berk et al. 2008) and unipolar (Berk et al. 2014) depressed patients.

2.4.6 Cholinergic super sensitivity hypothesis

The cholinergic super sensitivity hypothesis was postulated when it was found that central cholinergic activation has a negative effect on mood, while anticholinergic drugs or adrenergic stimulation induces behavioural activation and arousal. The data suggests that heightened cholinergic tone and decreased adrenergic tone could lead to depressive symptoms (Mineur et al. 2013). Secondly levels of acetylcholine are elevated in depressed patients (Mineur et al. 2013) and this cholinergic dysfunction may account for cognitive symptoms associated with depression, especially when the disease is long-lasting and treatment resistant (Dagytė, Den Boer & Trentani 2011). Acetylcholine is largely responsible for cognitive processes and therefore the optimal cholinergic tone is vital for optimal behavioural and brain function (Dagytė, Den Boer & Trentani 2011). Chronic stress induces a malfunction of the cholinergic system leading to cognitive impairments, often seen in depressed patients (Dagytė, Den Boer & Trentani 2011). In support to these findings research show that individuals exposed to irreversible cholinesterase inhibitors such as diisoprpylfluorophosphate (DFP) develop depressive symptoms through the increase of acetylcholine levels (Janowsky, Overstreet & Nurnberger 1994). Furthermore neuroimaging studies have found increased levels of choline, the precursor of acetylcholine, in depressed patients and its reversal after recovery from depression (Dagytė, Den Boer & Trentani 2011). It has been suggested that the hippocampus may be critical in mediating cholinergic effects on stress-related behaviours as increased cholinergic tone in the hippocampus leads to depressive-like symptoms (Mineur et al. 2013).

Animal models of depression have also been shown to be related to cholinergic signalling, adding support for the cholinergic super sensitivity hypothesis. One such model, the Flinders sensitive line (FSL) rat, was developed in 1982 (Overstreet 1993). Although originally bred to be resistant to organophosphates in order to try and understand the mechanism behind those who were tolerant to organophosphates, a strain genetically more sensitive to organophosphates was bred (Janowsky, Overstreet & Nurnberger 1994, Overstreet 1993, El Yacoubi, Vaugeois 2007). Surprisingly, it was also noted that these animals display exaggerated immobility in the forced swim test (FST) (i.e. depressive-like behaviour), which can be reversed by chronic (but not acute) treatment with antidepressants (El Yacoubi, Vaugeois 2007). It should be noted, however, that hypercholinergic activity is not the only trait of this rat line ascribed to its depressive-like behaviour and the FSL will be described in greater detail in §2.6 below.

Although the cholinergic super sensitivity hypothesis seems plausible, cholinergic hypersensitivity in humans has also been linked to a personality trait that predisposes to depression, such as stress sensitivity, rather than to depression itself. Furthermore, although scopolamine have been shown to display rapid anti-depressive effects (Drevets, Zarate & Furey 2013), no other anticholinergic drugs express consistent anti-depressive properties and thus no anticholinergic drugs have made the

antidepressant market. That said it appears that some classical antidepressants show downregulation of cholinergic neurotransmission, suggesting the involvement of acetylcholine, at least as contributing factor, in depression (Brink et al. 2004). The SSRI, citalopram for example, has been shown to reverse memory impairment by enhancing acetylcholine release in the hippocampus of laboratory animals, improve psychotic symptoms and behavioural disturbances in patients with dementia as well as improve memory consolidation in healthy volunteers (Dagytė, Den Boer & Trentani 2011). Fluoxetine, another SSRI, also displays efficacy in reversing the increased cholinergic tone in the hippocampus most likely via an increase in acetylcholine esterase (AChE) (Mineur et al. 2013).

2.5 Treatment

Multiple treatment strategies with varying degrees of effectiveness exist for the treatment of MDD. Strategies or interventions are subdivided as either pharmacological or non-pharmacological. Pharmacological interventions i.e. antidepressants are often used as first line therapy whereas non-pharmacological interventions such as psychotherapy, life-style adjustments and support groups are used as augmentation or in monotherapy of mild depression (Willner, Scheel-Krüger & Belzung 2013, Marais, Stein & Daniels 2009). Psychotherapy further includes psychosocial interventions and relaxation techniques, whereas life-style adjustments often include biophysical interventions such as dietary optimization and exercise. Although several options exist for both mild and moderate depression, pharmacotherapy remains the first-line therapy in moderate to severe depression (Willner, Scheel-Krüger & Belzung 2013). This is troublesome as only 30-35% of patients respond to treatment, 29-46% partially responds and 19-34% of patients do not respond to multiple treatment attempts (Marais, Stein & Daniels 2009). In addition, there are well documented withdrawal-like reactions upon termination of treatment, referred to as antidepressant discontinuation syndrome (Whittington et al. 2004). Discontinuation syndrome is a transient condition that occurs after sudden withdrawal or reduced dose of antidepressants and leads to bothersome symptoms, most often described as being flu-like (Warner et al. 2006). Furthermore, treatment of childhood depression is complicated, particularly due to vulnerability of juveniles and even more so since data on the safety and efficacy as well as pharmacological treatment options are limited. Furthermore there is a need to optimise treatment in both childhood and adulthood depression in order to reduce the risk of relapse or recurrence. In doing so, alternative options or effective augmentative strategies to pharmacotherapy are urgently sought (Marais, Stein & Daniels 2009). In the sections below pharmacotherapy in general and SSRIs in particular in the treatment of childhood depression will be discussed, followed by an exploration of non-pharmacological treatment strategies, in particular exercise.

2.5.1 Pharmacotherapy

All antidepressants currently on the market are classified according to the neurobiological target, in most cases related to their effect on the monoaminergic system (Willner, Scheel-Krüger & Belzung 2013):

1. Monoamine oxidase inhibitors (MAOI), *selegiline, tranylcipromine*
2. Tricyclic antidepressants (TCA), *amitrityline, *desipramine, imipramine*
3. Selective serotonin reuptake inhibitors (SSRI), *fluoxetine, escitalopram*
4. Serotonin and noradrenalin reuptake inhibitors (SNRI), *venlafaxine, duloxetine*
5. Noradrenalin reuptake inhibitors (NARI), *atomoxetine, reboxetine*
6. Dopamine reuptake Inhibitors, **bupropion, *desipramine*
7. $5HT_2$ Antagonists, *nefazodone, trazodone*
8. Tetracyclics and Unicyclics, *amoxapine, mirtazapine, *bupropion*
9. Atypical drugs, *agomelatine, tianeptine*
10. Herbals, *St John's wort*

(*drugs belonging to more than one class)

Even though a spectrum of antidepressants are available for the treatment of depression in adults, very few are effective and suitable for the treatment of childhood depression. In fact, the FDA has approved only fluoxetine for the treatment of MDD in children 7-12 years of age, and fluoxetine or escitalopram for the treatment of MDD in adolescents 12-18 years of age (Soutullo, Figueroa-Quintana 2013). The lack of treatment options has mainly been the result of the limited data available on the variety of medication used in children and adolescents due to children primarily treated as little adults in the past (Cheung, Emslie & Mayes 2005) as well as ethical implications in using children in randomised clinical trials. Additionally the research on the use of SSRIs in children has been troublesome as most trials testing the efficacy are of short duration, have small numbers and are industry sponsored, limiting the ability to detect or report major adverse events (Kastelic, Labellarte & Riddle 2000). **Fortunately in recent years the practice of treating children as small adults have changed with the FDA requiring paediatric data on all new compounds, as well as providing incentives to obtain data for existing medications** (Cheung, Emslie & Mayes 2005, Libby et al. 2007). The amount of data available on some drugs has consequently increased significantly. Yet, the controversy about the safety and efficacy of antidepressants, particularly SSRIs and SNRIs, has also caught the limelight, resulting in a "blackbox warning" issued in September 2004. This warning relates to increased suicidal ideation in children and adolescents under the age of

18 years during the first few months of treatment (Klomp et al. 2014, Libby et al. 2007). Nevertheless, there have been no reported cases of completed suicide attributed to the use of SSRIs in this age group (Soutullo, Figueroa-Quintana 2013).

However the SSRIs are currently the most prescribed antidepressants in all age groups, due to their relative safety and better side-effect profile as compared to the older TCAs and MAOIs (Cowen 2008, Klomp et al. 2014). As mentioned earlier only fluoxetine and escitalopram are approved by the FDA for treatment in children (Oberlander, Miller 2011). Additionally SSRIs are also the only drugs that show efficacy in this age group according to meta-analyses and randomised control trials (Thapar et al. 2012). The effectiveness of SSRIs over TCAs are ascribed to different maturation rates of monoaminergic pathways in the developing brain as described in par 2.7. The use of SSRIs in the treatment of childhood depression is further discussed below.

2.5.2 SSRIs in the treatment of childhood depression

Fluoxetine and escitalopram are SSRIs, which inhibit the serotonin transporter (5HTT) protein (Figure 2-4) (Willner, Scheel-Krüger & Belzung 2013). In this way the synaptic reuptake of serotonin from the synapse into the presynaptic neuron is inhibited, resulting in an increased synaptic concentration of serotonin (Baldessarini 2006) as its release is no longer accompanied by transport back to where storage vesicles are located (Walker 2013). The acute effects of SSRIs are well defined and although they rapidly increase the brain's serotonin neurotransmission, they exert their mood-elevating effects only after prolonged administration (Iñiguez, Warren & Bolaños-Guzmán 2010, Kovačević, Skelin & Diksic 2010, Stahl 1998, Harmer, Cowen 2013). Alleviation of symptoms is only experienced 2-4 weeks after the start of treatment and treatment duration needs to last even longer (several months) for complete remission (Berton, Nestler 2006).

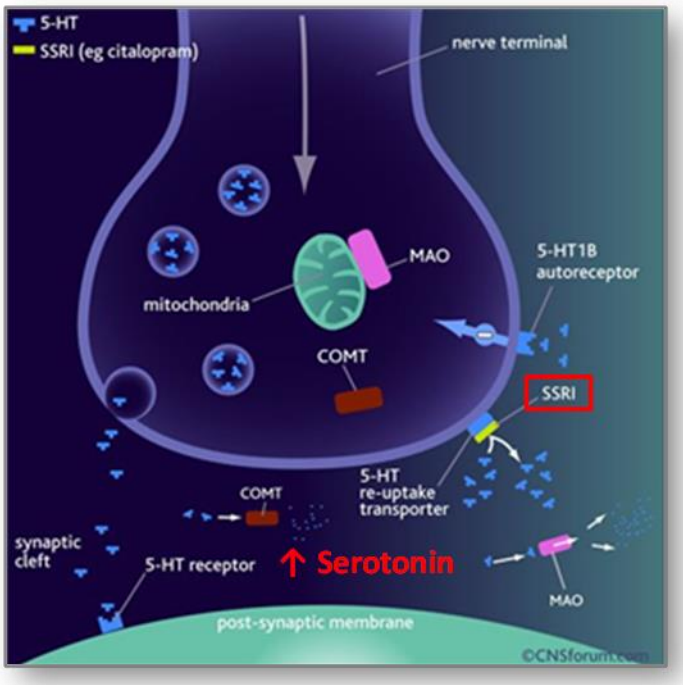

Figure 2-4: SSRIs blocks serotonin reuptake, thereby increasing the concentration of serotonin in the synapse; adapted from (Rang et al. 1995)

SSRIs also show anti-inflammatory action (Walker 2013) and has been shown to decrease the inflammatory cytokines IL-1 and IL-6 (Hannestad, DellaGioia & Bloch 2011) and increase the anti-inflammatory cytokine IL-10 (Janssen et al. 2010), without influencing levels of TNF-∝ (Hannestad, DellaGioia & Bloch 2011, Eller et al. 2008). SSRIs have also been shown to display anti-oxidative effects, such as a significantly decreasing MDA and SOD in human patients using fluoxetine (Khanzode et al. 2003, Bilici et al. 2001). Upon further investigation it was demonstrated that serotonin can activate downstream signalling pathways and activate transcription factors that eventually influence the expression of proteins associated with the regulation of neural plasticity, stress resistance and cell survival in adults of both humans and rodents (Marais, Stein & Daniels 2009, Harmer, Cowen 2013, Jin et al. 2009). The 5HTT itself is also of interest as it is the primary target of SSRIs and appears to have a critical function in regulating emotion. A genetic variant which has been shown to reduce the expression of the 5HTT has been associated with neuroticism, anxiety-like behaviour and depressive symptoms (Ansorge et al. 2004). Lastly, although having a very low affinity for dopamine receptors or dopamine transporters, SSRIs have both acute and chronic effects on dopaminergic function. This has been ascribed to the property of serotonergic stimulation in various brain regions to potently release dopamine, eventually resulting in an antidepressant effect (Renard et al. 2001). However fluoxetine has also been found to decrease dopamine transmission via the down-regulation of the $5HT_{2C/2B}$ receptor (Dailly et al. 2004). Dopamine neurotransmission is therefore inhibited until tolerance to the action of serotonin on the $5HT_2$ receptors is induced (Dailly

et al. 2004). This indirect effect of SSRIs, and in particular of fluoxetine, on dopamine transmission could partly explain the delay before onset of therapeutic effect (Karanges, McGregor 2011, Dailly et al. 2004). As mentioned earlier, dopamine plays a major role in the anhedonic traits associated with depression and several studies have found an increase in dopamine transporters following chronic SSRI treatment (Rominger et al. 2015).

Since long-term treatment (months or even years) is necessary with antidepressants, it is not difficult to conceive that juvenile treatment may have a significant impact on neurodevelopment, influencing neurobiological functioning later in life (Iñiguez, Warren & Bolaños-Guzmán 2010). Enduring effects of juvenile fluoxetine exposure on neuroanatomy have been documented and is consistent with the ‘‘equal, but opposite’’ hypothesis. This hypothesis originates from observations in adult animals, where chronic drug exposure results in an accommodation to the drug effects, culminating from a series of compensatory reactions whereas chronic drug exposure in juvenile animals leads to assimilation by incorporating drug-induced changes in the form of permanent developmental alterations of the system (Andersen, Navalta 2004). Chronic exposure to fluoxetine prevents the normal development of dendritic spines in the hippocampus, as spine density in adulthood of neonatally treated rats was approximately equal to the density present when they had completed treatment as juveniles, suggesting that further spine development was arrested (Norrholm, Ouimet 2000). Prenatal fluoxetine exposure also decreased the density of the serotonin transporter (Montero, De Ceballos & Del Rio 1990), cortical phosphoinositide hydrolysis, and serotonin content in the cortex of pre-adolescent rats (Andersen, Navalta 2004). In contrast, another study found that fluoxetine produces an increase in serotonin levels in the frontal cortex after withdrawal (Sarkissian et al. 1990). Taken together, juvenile exposure to SSRIs could cause lasting decrements in the serotonin system in adulthood, although the effects of long-term SSRI treatment following withdrawal in adulthood have produced variable results that are highly dependent on the brain region examined (Andersen, Navalta 2004). Behavioural changes are also observed in adolescent animals after chronic SSRI treatment, when compared to a control group. These changes included altered body weight, reduced sexual functioning, increased anxiety-like behaviour (de Jong et al. 2006), unexpected risks of affective dysfunction later in life via the inhibition of the 5 HT-transporter (Ansorge et al. 2004), as well as influencing responsiveness to rewarding and aversive stimuli in adulthood (Iñiguez, Warren & Bolaños-Guzmán 2010). These complex functional outputs are likely regulated by many factors, including the emotional valence of the stimulus, the environment in which it is experienced, and the brain circuitry likely being engaged by it. Findings also demonstrate that fluoxetine-induced anxiety-like behaviour can be alleviated by re-exposure to fluoxetine itself (Iñiguez, Warren & Bolaños-Guzmán 2010).

Still, medical doctors are faced with the daunting task of prescribing antidepressants to children without really knowing the long-term consequences of prescribing during certain periods of

development such as the pre-pubertal period that is mostly overshadowed by research during neonatal and adolescent development, as well as the realization that no single treatment is effective for everyone. This highlights the need of finding alternative options or augmentation strategies with improved safety (Marais, Stein & Daniels 2009). Lastly, even though there are risks involved in early-life treatment it is important to optimise treatment in this age group to reduce the risk of relapse or recurrence.

2.5.3 Non-pharmacological interventions

In addition to serious pathology and sometimes unsatisfactory pharmacotherapy (including delayed onset of action, high rate of relapse and low efficacy of antidepressants), MDD has also been associated with other poor health indicators such as smoking, physical inactivity and high caloric intake (Abildgaard et al. 2011, Bonnet et al. 2005). Consequently research into new drugs as well as augmentation strategies for both the treatment of the disorder, as well as the reduction in risk factors for developing MDD, has enjoyed a lot of attention in recent years. The American Psychiatric Association (APA) recommends augmentation strategies in adults to only be considered after 4-8 weeks of inadequate response to initial therapy (Gersing et al. 2014). Augmentation includes the addition of another first-line antidepressant or second generation antipsychotics as well as non-pharmacological augmentations that include psychotherapy, electroconvulsive therapy, deep brain stimulation, sleep deprivation and lifestyle modifications. However, not all of these augmentation strategies are a therapeutic option in the treatment of childhood depression. Nevertheless, strategies such as psychotherapy, support groups or lifestyle related strategies can be employed. In particular, cognitive behavioural family-focused therapies have been shown to be effective in children (Hoagwood et al. 2014). Furthermore, dietary optimisation and exercise also seems to be plausible augmentation strategies, although less data is available on their efficacy in children.

Cognitive behavioural therapy as an intervention, aims to reduce symptoms, improve functioning and evidently remission of the disorder by involving the patient in a collaborative problem-solving process to test and challenge the validity of maladaptive cognitions and to modify maladaptive behavioural patterns (Hofmann et al. 2012). However, similar to antidepressants, cognitive behavioural therapy has relatively low remission rates and positive responders often have a high rate of relapse. Cognitive behavioural therapy is also expensive and often inaccessible (Brown et al. 2013).

Dietary augmentation strategies include dietary restriction such as caloric restriction and omega-3 polyunsaturated fatty acids supplementation. Notably the beneficial effects of caloric restriction are dependent on age and duration of food restriction and can therefore also negatively impact on behaviour (Ota, Duman 2013). However a genetic predisposition also predicts higher response to develop depression as it was found that high-fat diets can exacerbate depressive-like behaviour in rodents with a predisposition to develop depression (FSL), whereas control rats (FRL) did not develop

depression despite of an higher caloric intake (Abildgaard et al. 2011). Omega-3 polyunsaturated fatty acids also show great promise in alleviation of depressive symptoms, even though results are mixed and the independent efficacy of these fatty acids remains to be elucidated. That said, it has been found that supplementation with omega-3 polyunsaturated fatty acids started concurrently with SSRIs can enhance the efficacy of SSRIs compared to placebo (Ota, Duman 2013).

A sedentary lifestyle is a well-known risk factor for cardiometabolic diseases (Griffin et al. 2011, Dishman et al. 2006), and has also been shown to be a risk factor for the development of MDD (Carek, Laibstain & Carek 2011). Data suggest that physical activity may prevent depression and reduce the risk factors of diseases commonly co-morbid with MDD, such as cardiovascular disease (Eyre, Baune 2012). Exercise as an augmentative strategy in the treatment of MDD has been extensively studied in adults. However, similar to data on antidepressant treatment, these findings have to be extrapolated to children. Exercise has become a more acceptable strategy above antidepressant and cognitive behavioural therapy, most likely due to the stigma connected to pharmacotherapy and behavioural therapy (Carek, Laibstain & Carek 2011). Studies of exercise in adults with MDD have demonstrated that exercise as monotherapy, combination therapy and augmentation with antidepressants may be as effective as medication and psychotherapy (Carek, Laibstain & Carek 2011), thereby supporting the rationale of conducting studies of exercise as treatment option in children. There are indications that physical activity may have a positive effect on mental health in children (Camero et al. 2012), although the hard evidence-base is lacking (Dopp et al. 2012). Consequently, investigations leading to stronger evidence are needed for the use of exercise in the treatment of childhood depression. Exercise as a possible augmentation strategy will be described in greater detail in par. 2.5.3.1 below.

2.5.3.1 Exercise

Physical activity refers to any bodily movement produced by skeletal muscles that increases energy expenditure, including both spontaneous physical activity and voluntary exercise (Seo et al. 2014). Physical exercise is therefore defined as a subset of physical activities that are designed as part of a goal-oriented program (Carek, Laibstain & Carek 2011). It can, for example, be designed to counter pathophysiological processes, thereby to prevent or manage numerous chronic conditions (Seo et al. 2014). Worryingly, although the beneficial effects of exercise are well recognised, only about 30% of the Western population engage in a sufficient amount of exercise on a weekly basis to promote or sustain general health, and once started roughly half of participants stop within 3-6 months (Salmon 2001).

Structured exercise can be divided into aerobic and non-aerobic exercise. Aerobic exercise such as running and swimming which involves prolonged activity of large muscle groups, has received tremendous attention in recent years due to their integral role in cardiovascular conditioning programs

(Doyne et al. 1987). Anaerobic exercise, such has weightlifting, strength training, coordination and flexibility, has received less attention due to the nature of the exercise which involves brief intense muscular activity that is usually unsustainable and since it is not associated with the same cardiovascular benefits as aerobic exercise (Blumenthal et al. 2007, Doyne et al. 1987). Although aerobic exercise is favoured, both aerobic and non-aerobic exercise has been found to be effective in the reduction of depressive symptoms, as reported in a study conducted in adult depressed women that found no significant differences between the anti-depressive effects of running and weightlifting compared to control groups (Doyne et al. 1987). These findings suggest that the antidepressant effects are not dependent upon the aerobic effect of exercise (Doyne et al. 1987). Interestingly, fitness as a general concept seems to play a role. Even fit individuals who were deprived of exercise experienced worsening of their mood and increased anxiety (Ströhle 2009).

The aforementioned sustainability of aerobic exercise and its effect on general health and wellbeing form the basis of the idea that exercise may play an important role in the treatment of depression and is supported by findings that group exercise training in the elderly is as effective as sertraline (Ströhle 2009). That said the therapeutic effects of exercise on depressive-like behaviour have been most studied in adults and the elderly (Cotman, Berchtold & Christie 2007), although one cross-sectional study, found a correlation between habitual exercise and lower incidence of depression in adolescents (Ströhle 2009). These findings are also well supported by pre-clinical studies suggesting exercise to be effective in the alleviation of depressive-like behaviour in rodents (Blumenthal et al. 2007) and in addition that the age of the subject is of great importance when interpreting results. It has been found in pre-clinical studies that low intensity exercise results in beneficial effects between PostND21-30 (pre-puberty), whereas high intensity seems to be more beneficial for neuroplasticity in PostND31-40 rats (adolescence) (de Almeida et al. 2013). Brain neuroplasticity decreases as age progresses, so that it is important to assess whether exercise alters plasticity during early life and to determine the basic mechanism of such effects (Gomes da Silva et al. 2012).

In humans exercise is regarded a relatively safe, affordable and readily available therapeutic option, with some evidence even suggesting that it may be an effective first-line treatment in mild to moderate depression (Carek, Laibstain & Carek 2011, Brown et al. 2013, Marlatt, Lucassen & van Praag 2010), although it is not included in the guidelines of the American Psychiatric Association (APA 2006). Exercise has therefore been proposed to be used to augment the effect of antidepressants. A study done in elderly patients who have not responded to treatment in 6 weeks, showed a significant improvement in symptoms when exercise was added, as compared to non-exercised social controls (Carek, Laibstain & Carek 2011, Mather et al. 2002). These data spurred the idea that exercise may also be implemented as an augmentation strategy together with antidepressant treatment, with the potential of enhancing the effectiveness of antidepressant treatment, or even to compliment antidepressant treatment in partial responders or patients presenting with treatment-

resistant depression (Russo-Neustadt et al. 2004, Trivedi et al. 2006). Although most research has been done in adults, similar outcomes may potentially be attainable in children and adolescents (Brown et al. 2013).

The potential of exercise to alter mood and stress responses has been ascribed to several mechanisms, as observed in both clinical and pre-clinical studies (Marais, Stein & Daniels 2009). The mechanism of action is quite similar to that of antidepressants, so that exercise may induce both antidepressant and anxiolytic effects (Marlatt, Lucassen & van Praag 2010). The efficacy of exercise in the treatment of depression may be associated with one or more of the following mechanisms (Eyre, Baune 2012, Marlatt, Lucassen & van Praag 2010, Bjørnebekk, Mathé & Brené 2010):

- Altered monoamine metabolism by increasing neurotransmitters particularly serotonin in the central nervous system
- Decreased long-term basal levels of cortisol (HPA axis function)
- Increased neurotrophins such as BDNF, neurogenesis, angiogenesis, dendritic spine density and synaptic plasticity
- Decreased pro-inflammatory cytokines (IL-1β, IL-6, TNF-α)
- Increased anti-inflammatory cytokines (IL-10)
- Modulate the release of glutamate
- Reduction of reactive oxygen species (ROS)
- Reduction in markers of oxidative stress

Although exercise seems to be a favourable therapeutic option, no therapeutic administration of physical activity to patients with depression has been developed (Ströhle 2009). Usually three to four training sessions per week lasting 30 to 45 minutes for 10 to 12 weeks are recommended (Carek, Laibstain & Carek 2011). Furthermore, interventions tailored to the individual seem to be more effective than generic interventions; however such a variety in programs and combinations renders the prescription of exercise in the treatment of patient with depression specialised and challenging. It is of note that exercise-induced neuroplastic effects are dependent upon exercise intensity, as well as developmental age (de Almeida et al. 2013, Gomes da Silva et al. 2012), which should also be accounted for. Prescribing exercise at a singly defined intensity is something seen quite often in scientific journals, intended to simplify the estimation of exercise intensities that would produce equivalent effects in individuals with different absolute exercise capacities. In short it means that individuals are prescribed exercise intensities as a percentage of their maximal oxygen uptake (VO_2max) or maximum heart rate (HR_{max}) in order to individualize exercise. Thereby these parameters account for differences in physiological and functional capacity by producing equivalent

exercise stress in individuals despite their difference in phenotype. Although VO_2max and HR_{max} are not the only parameters used, they are the most common, described in the literature. Studies implementing other parameters have produced much less consistency in outcome (Mann, Lamberts & Lambert 2013).

It was in found in meta-analysis that high intensity exercise has a higher effect size in clinically depressed adults, as compared to the overall population (non-clinical) (Rethorst, Wipfli & Landers 2009). However, the effect of moderate intensity exercise was found to be significantly lower than that of high intensity and low intensity exercise, with no significant difference between high and low intensity (Rethorst, Wipfli & Landers 2009). That said, none of the exercise intensities exerted a significant decrease on depressive symptoms when compared to the control clinically depressed population (Rethorst, Wipfli & Landers 2009). Still, findings of the treatment duration, frequency of treatment, exercise type and the exercise intensities have been used to formulate the guidelines of the UK National Institute for health and clinical excellence (NICE) for the treatment of depression in adults (Stanton, Reaburn 2014). Furthermore, it is generally accepted that low and moderate intensity exercise produce beneficial effects in the brain, whereas high intensity may be harmful (de Almeida et al. 2013). Surprising, as a recent Cochrane review was unable to perform analysis on exercise intensity due to methodological differences between studies and a lack of clear intensity parameters (Larun et al. 2006). This may explain the lack of consensus regarding low, moderate and high intensity exercise for the treatment of depression (Carek, Laibstain & Carek 2011) and necessitates further exploration. Inconspicuously, clinical studies have found that reaching a sufficient intensity is more important than the type of exercise performed (for example running versus strength training) (Carek, Laibstain & Carek 2011). This idea would support previous findings that aerobic and resistance exercise may be of similar benefit, but still without consensus on a specific intensity. Based on the previously mentioned findings we propose that the earlier mentioned statement on reaching a sufficient intensity independent of exercise type should be revised to reaching a sufficient intensity specific to age during development is more important than the type of exercise performed

In summary, it has been postulated that during development the brain becomes wired to match the needs of or compensate for its environment. Both chronic drug exposure and exercise represent important environmental modulators in this scenario. As a result, exposure to psychoactive drugs or exercise, particularly during vulnerable periods of development, may produce changes in neurodevelopment that would be reflected in subtle, or possibly obvious, differences in mature response to the environment (Murrin, Sanders & Bylund 2007). Importantly, such plastic effects of the brain are specifically found during a pre-pubertal window of opportunity (Andersen, Navalta 2004) as described in greater detail in par 2.7.

2.6 Animal models of depression

Studies using animals have been used for centuries in order to better understand the mechanism of human disease. Such models allow for studies under controlled conditions (Andersen, Navalta 2011), particularly where human studies pose very challenging ethical dilemmas, non-withstanding the ethical dilemmas that animal studies in itself face. Animals can be subjected to more invasive procedures that are rarely used in children and adolescence, as well as in studies that extend to molecular level (Andersen, Navalta 2011). Animal models can also be implemented to examine drug effects, without complications of drug-drug interactions from multiple drug use typically seen in clinical trials. This is particularly important, as depressed individuals often seek secondary treatment options (Andersen, Navalta 2011). The studying of long-term effects may be more feasible in animals due to their shorter life cycles. This may become a tremendous advantage, particularly as non-adherence during chronic drug-therapy becomes a challenge in humans. There is also a long delay between childhood interventions and the eventual long-term effects thereof in adulthood, so that results can be obtained only after decades, as opposed to weeks in some animal models (Andersen, Navalta 2011).

Although animal models can provide valuable insight into the basic mechanism of disease and drug action, data should eventually be translatable to the human disease or drug action in humans. Animal studies are therefore not without limitations and these limitations should be accounted for when interpreting the data. Such limitations can be ascribed to inter-species differences, the complexity of brain development and the alignment of comparable developmental periods between animals and humans for comparison and therefore ensuring translatability of findings. However these processes in mammals are often remarkably similar, allowing for extrapolation to humans. This includes brain development (Andersen, Navalta 2011).

However, any translational animal model of disease needs to be validated and should meet certain criteria. Validation criteria were originally defined and set up by McKinney and Bunny (McKinney, Bunney 1969), which are still used today, and states that the model should have:

- Construct validity – *the ability of the model to replicate neurobiological factors*
- Face validity – *the model's symptomatic homology to the human disorder/disease*
- Predictive Validity – *the animals respond to the same treatments that humans respond to, and conversely do not respond to treatments that humans do not respond to .*

In short this means that to be a valid model of a specific disorder, the animals should display similar pathophysiology, similar symptoms, respond to similar treatment as the human condition (El Yacoubi, Vaugeois 2007) and should also not respond to treatment that is ineffective in treating the human disorder. Animal models of MDD and anxiety disorders have successfully been employed to better

understand the neurobiology of depression and to predict successful treatment strategies (Malkesman et al. 2006). Several animal models of MDD and anxiety disorders that have been established include (Overstreet 1993):

- Wistar Kyoto rats
- Swim high-active and swim low-active rats
- Congenitally learned helpless and congenitally non-learned helpless rats
- Fawn-hooded rats
- High and low reaction to stress test mice
- Flinders sensitive line (FSL) and resistant line (FRL) rats as the control

Yet, no animal model of depression captures the periodic change of behaviour into and out of depression as seen in patients with depression (Belmaker, Agam 2008).

The FSL as an animal model of depression was developed in 1982 (Janowsky, Overstreet & Nurnberger 1994), originating from a breeding programme that intended to create a strain of rats that was genetically resistant to the effects of the organophosphate anticholinesterase agent diisopropyl fluorophosphates (DFP). One line of rats was genetically more sensitive to DFP due to an increase in muscarinic receptors in several brain regions, consequently named the FSL rats (Overstreet et al. 2005). A second line, the FRL rats, does not display this cholinergic supersensitivity, thereby acting as control (Overstreet et al. 2005). It has been observed before that depressed humans were more sensitive to cholinergic agonists than normal controls (Janowsky et al. 1972). The FSL rat line resembled depressed humans with regard to the cholinergic supersensitivity, which raised the possibility that the FSL rats might be a model of depression. This eventually led to investigations demonstrating that the FSL rat also displays depressive-like behaviour in the FST, as well as pronounced serotonergic abnormalities, consistent with the neurobiology of depression (Overstreet et al. 2005, Overstreet et al. 1994). The FSL rat is a more sensitive genetic translational animal model for depression, displaying cholinergic super sensitivity which is believed to contribute to its depressive-like behaviour (Overstreet et al. 2005). The FSL rat show reduced locomotor activity and body weight as well as an increased REM sleep and cognitive (learning) difficulties (Overstreet et al. 2005). Other behavioural features of the FSL rat line which are related to depression include changes in appetite, psychomotor function, sleep patterns and immune function, altered sleep patterns and lower serotonin levels (El Yacoubi, Vaugeois 2007). Another important characteristic of the FSL rat relates to its unique predictive validity, where these rats display antidepressant response in the FST following chronic but not acute treatment with antidepressants, similar to that observed in humans (Cryan, Markou & Lucki 2002, Overstreet et al. 2005). Furthermore these animals also do not respond to treatments that are not effective in the human condition, as seen with the administration of

benzodiazepines used for anxiety without having antidepressant effects (Cryan, Valentino & Lucki 2005). All of these taken together suggest that the FSL rat is indeed a unique rodent model for depression (Overstreet et al. 2005, Overstreet, Wegener 2013).

However suicidal ideation cannot be modelled in the FSL rat. Also, the FSL rat does not display anhedonic traits, no cognitive disturbances and no disturbances in slow wave sleep as seen in depressed individuals (Overstreet et al. 2005). It is, however, important to consider that a model never fully describe the human condition it models.

Prepubertal FSL rats display significantly longer immobility times than controls, suggesting that these rats may also represent a useful model of childhood depression (Malkesman et al. 2006). FSL rats also exhibit much higher levels of social play (chasing, boxing and attacking) than their controls, which seems in contrast to the absence of normal play, antisocial behaviour, avoidance and withdrawal as seen in clinical symptoms of childhood depression (Malkesman et al. 2006). However, behaviour such as climbing, boxing and attacking can also be seen as aggressive behaviour which is often seen in children with depression (Braw et al. 2006). FLS rats also show lower anxiety levels than FRL controls, as seen in the OFT with pre-pubertal rats spending more time in the centre of the open field, or in the elevated plus maze (EPM) with FSL rats spending more time in the open arms (suggestive of decreased anxiety) (Braw et al. 2006). Adult FSL rats show a different anxiety-like behavioural profile than their pre-pubertal counterparts with normal anxiety levels in tests such the EPM but exhibit anxiogenic behaviour in the social interaction test and active avoidance task (Overstreet et al. 2005). Pre-pubertal FSL rats exhibits lower basal levels of corticosterone and ACTH which seems to persist into adulthood, albeit the levels increase slightly towards adulthood (Malkesman et al. 2006). That said hypercortisolism is one of the most robust phenomena seen in major depression (Lopresti et al. 2014). At the same time, as a paradoxical finding, severe stress in humans seems to result in hypocortisolism (Heim, Ehlert & Hellhammer 2000).

Based on the above findings it could be said that the pre-pubertal FSL rat is a suitable model for paediatric and childhood depression and more specifically depression with low levels of anxiety.

2.7 Neurodevelopment

MDD is known to be a neurodevelopmental disorder that results from an interplay between genetic and environmental factors (Kessler, Avenevoli & Ries Merikangas 2001). These affect the maturation of brain circuits involved in affective functioning, ultimately leading to depressive disorders in adulthood (Ansorge, Hen & Gingrich 2007). The role of development and the importance of these neurodevelopmental processes have therefore been recognized in the aetiology of MDD and could assist in better understanding this disease (Hankin 2015). Furthermore, that many antidepressants are ineffective in children and that only SSRIs have shown efficacy in this group, has been explained by

the different maturation rates of the different monoaminergic systems, i.e. that the serotonergic system develops much faster than the noradrenergic and dopaminergic systems (Murrin, Sanders & Bylund 2007). Furthermore, the developing brain is ever changing and adapting via several mechanisms (Andersen, Navalta 2004), as discussed in greater detail below, making it even more vulnerable to external influences.

In humans prenatal development occurs over a period of 40 weeks divided into three trimesters (Murrin, Sanders & Bylund 2007). In rodents prenatal development occurs over a 21-day gestational period, which correlates neurologically to the first and second trimester of human development as seen in (Figure 2-5). The third trimester in human pregnancy correlates to the neonatal or pre-weaning stage in rodents (Eiland, Romeo 2013), a stage known as a protracted period of parental care after birth, lasting 14–21 days. The pre-pubertal period in rodents happens when pups are weaned from the dams at 21 days of age, corresponding neurologically to roughly 4 years of age in humans. Rats reach sexual maturity at about 5 weeks of age, which corresponds to puberty or early adolescence in humans (Murrin, Sanders & Bylund 2007, Eiland, Romeo 2013). Although the specific age-span that covers adolescence in male and female rats are not entirely clear cut, animals between 30 and 60 days of age undergo behavioural and neurobiological transformations similar to those observed in other species during adolescence, including humans (Eiland, Romeo 2013) reaching early adulthood by PostND60.

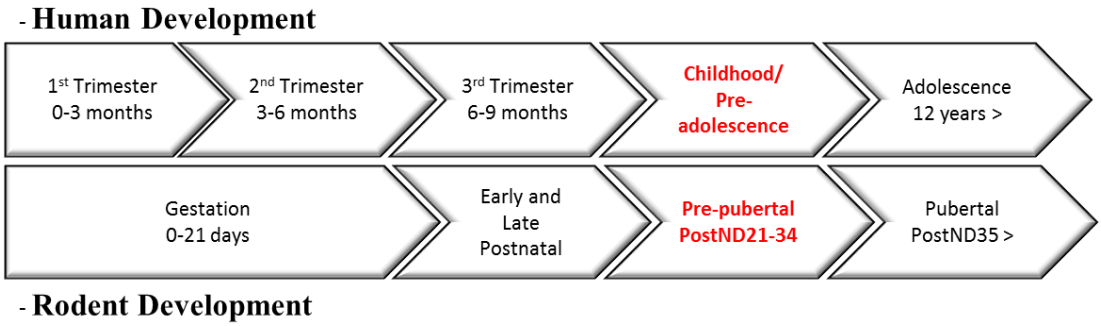

Figure 2-5: Human versus rodent development, adapted from (Kepser, Homberg 2015)

Research comparing rodent and human neurodevelopment has found confounding similarities and alignment of age-related brain development. Secondly, hormonal changes also 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 of great importance when interpreting data (Murrin, Sanders & Bylund 2007).

2.7.1 Brain development

It has been demonstrated that the weight of the rat brain relative to bodyweight at birth is comparable to that of the human brain by the second trimester (Murrin, Sanders & Bylund 2007). Brain

development occurs in multiple stages in the rodent for example: the somatosensory system develops in early postnatal life (PostND0-14), sexual differentiation occurs between PostND7-20, synaptogenesis peaks by PostND21 (Andersen 2003), the cortex, hippocampus, amygdala and the cerebellum that continues to mature during puberty and adolescence at different developmental trajectories (Eiland, Romeo 2013) and therefore these neurodevelopmental changes set the stage for differential periods of vulnerability in a regionally specific manner (Andersen 2003). The substantial structural and functional remodelling of the brain, particularly within limbic and cortical regions occurs during adolescent development. For instance, human and non-human animal studies have shown significant volumetric increases in the hippocampus and amygdala in the early stages of puberty. Longitudinal studies combined with structural neuroimaging techniques have also revealed dynamic cortical gray- and white-matter volume changes throughout adolescence. Specifically, increases in frontal and temporal cortical volumes are observed from childhood to the onset of puberty, which is then followed by a period of cortical thinning during adolescence and into young adulthood (Eiland, Romeo 2013).

Brain development has been described by the concept of "use it or lose it". In this regard the brain overdevelops at first and is then refined to what will be needed when matured. Therefore, abundant and unused cells are constantly being removed, a phenomena that is in part regulated by serotonin (Whitaker-Azmitia 2001). Furthermore structural and functional changes occur in the brain throughout the lifespan, including the ability to change structure and function, known as plasticity (Andersen 2003). Plasticity allows the central nervous system to acquire new information and learn new skills, to reorganize neuronal networks in response to environmental stimulation, and to recover from brain injuries (Gomes da Silva et al. 2012). Brain development is a complex process and stimuli during this period could determine the brain's functional integrity in adulthood (Gomes da Silva et al. 2012). For example, social isolation during juvenile and adolescent stages can profoundly affect pre-frontal cortex functioning, by disrupting synaptic plasticity and decrease dopamine and serotonin signalling (Baarendse et al. 2013). Many of these changes have also been linked to the hippocampus, a brain region implicated in learning, memory and emotional processes and highly susceptible to neurodegenerative disease (Baarendse et al. 2013). Synaptic plasticity, as mentioned earlier, is regulated by growth factors throughout the lifespan and plays a significant role in neuronal changes associated with learning, drug exposure and neuronal repair following injury or transplantation (Andersen, Navalta 2004). These innervations are guided by neurotrophic factors, which keep on playing an integral role in dendritic branching when neurons reach their target. During the prenatal phase the expression of growth factors reach their highest level as neurons first establish their synaptic contacts. Growth factors rise again in a regionally specific manner during postnatal development of the rat. BDNF mRNA reaches an adult level in the hippocampus by day 7 and

remains elevated throughout juvenile development (Andersen 2003). Cortical levels however peak at 14 days of age and declines gradually (Andersen 2003).

2.7.2 Neurotransmitters

The serotonergic, noradrenergic and dopaminergic systems are implicated in the pathophysiology of depression. In addition, the stage of maturation of these systems significantly affect the efficacy of drugs administered during the different developmental stages, as described in greater detail below.

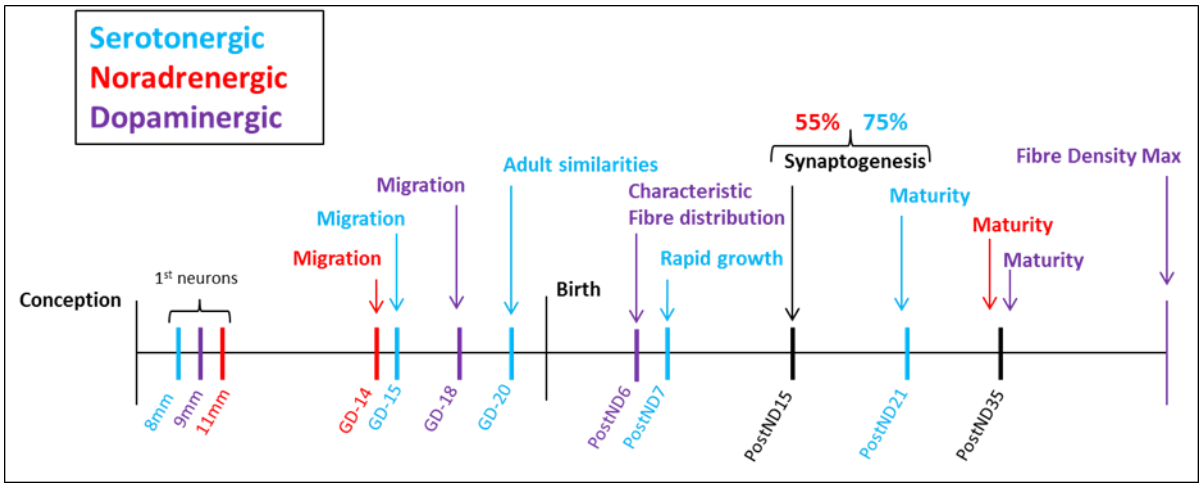

Figure 2-6: Schematic representation of the age-related neurodevelopment in the rodent, adapted from (Steyn 2011)

2.7.2.1 Noradrenergic development

In the human brain, tyrosine hydroxylase, the rate limiting enzyme for nor-epinephrine synthesis, can be detected by 4 weeks of gestation (Murrin, Sanders & Bylund 2007). In the rat embryo, norepinephrine containing neurons are detectable in the 11 mm embryo (Figure 2-6) and the noradrenalin neurotransmitter is already detectable at around 5-6 weeks of gestation (Murrin, Sanders & Bylund 2007). In rats, norepinephrine neurons differentiate between gestational days 10-13. Synthetic enzymes for norepinephrine and epinephrine are expressed when all of the noradrenergic cells appear to be present. The levels of norepinephrine in humans correlates with that of the rat, as it increases throughout the first trimester, especially from 2 months of gestation where after a decrease of 30-40% occurs between 6 months and early childhood (Murrin, Sanders & Bylund 2007). Monoamine neurons are detectable by gestational day 13-18 in rats (Andersen 2003). Migration of cortical neurons begins at GD14 and continues throughout early postnatal development (Figure 2-6) (Murrin, Sanders & Bylund 2007). Maturation of cortical neurons occurs largely within the first 3 weeks of postnatal development, the same period during which norepinephrine transporters reach adult levels (Murrin, Sanders & Bylund 2007). It is also during this period where noradrenergic innervation is increasing to adult levels but is only established in adulthood patterns by the fourth or

fifth postnatal week (Murrin, Sanders & Bylund 2007). Due to the later development of the noradrenergic pathway it has been identified as the main culprit in the ineffectiveness of TCAs, MAOIs and SNRIs in the treatment of children although they appear to be effective in adults (Murrin, Sanders & Bylund 2007).

2.7.2.2 Dopaminergic development

Dopamine is synthesised from phenylalanine and tyrosine in the cytoplasm of presynaptic neurons (Murrin, Sanders & Bylund 2007). Neurons containing dopamine are already present in the 9 mm rat embryo (Figure 2-6) and differentiate at approximately 10-15 days of gestation (Andersen 2003). In the human foetus dopamine neurons are present from 6-8 weeks (Murrin, Sanders & Bylund 2007). The dopamine turnover is relatively high during the perinatal period compared to adults (Herlenius, Lagercrantz 2004). Dopaminergic markers, including tyrosine hydroxylase activity, dopamine uptake sites and dopamine content attain adult levels between PostND28 and PostND35 (Andersen 2003). Studies on dopamine receptors show a steady increase in concentration of receptors with a peak at PostND35 to PostND40 followed by a decline between PostND35 to PostND60 to adult levels (Rho, Storey 2001).

Dopamine plays a role in psychiatric diseases such as schizophrenia, parkinsons as well as depression (Randrup et al. 2013). Dopamine has been implicated in coping with sudden changes in task requirements and therefore alteration in the development of dopaminergic pathways could lead to several long-term effects. For example, the effects of social isolation as a developmental insult (PostND21-42) are attributed to dopaminergic mechanisms as the persistent alterations during a vulnerable phase of development leads to marked changes in social behaviour as well as disrupted impulse control, impaired decision making and cognitive control deficits in adulthood (Baarendse et al. 2013). More specific were a loss of sensitivity to dopamine in the medial prefrontal cortex, alterations in dopamine and serotonin signalling and disrupted synaptic plasticity increasing the vulnerability to develop psychiatric disorders (Baarendse et al. 2013).

2.7.2.3 Serotonergic development

Serotonin synthesis depends on the dietary intake of tryptophan (Kepser, Homberg 2015). In the early phases of development the placenta secretes serotonin and supplies the brain with tryptophan as it will only start to synthesise serotonin in later stages. Tryptophan is transported over the blood brain barrier, where it is actively taken up in to serotonergic neurons (Maes et al. 2011). Serotonin is one of the first neurotransmitters present in the mammalian brain, and therefore plays a regulatory role in brain development before it assumes its role as a neurotransmitter (Mazer et al. 1997). Serotonin also plays a major role in neural plasticity and therefore any alterations in the serotonin system during the

stages of neurodevelopment can cause different developmental effects as different processes takes place during specific stages (Kepser, Homberg 2015).

According to Murrin and colleagues (2007) the 8mm rat embryo already has serotonin containing neurons (Figure 2-6) (Murrin, Sanders & Bylund 2007). In humans serotonin is evident by the 5th week of gestation, in rodents it is evident by gestational day 13, between gestational day 14 the serotonergic neurons have been formed and they grow towards the hippocampus, amygdala and prefrontal cortex and by gestational day 19 they are distributed in groups throughout the brain in a similar fashion to that seen in adults (Murrin, Sanders & Bylund 2007). The serotonin transporter is also transiently expressed throughout the brain from gestational day 14-15 till two weeks after birth (Murrin, Sanders & Bylund 2007), this transient expression is most likely to maintain serotonin at a specific level to steer developmental processes. Serotonin is implicated in the following developmental processes (Kepser, Homberg 2015):

- Promoting neurite outgrowth
- Synaptogenesis
- Differentiation/organization
- Neurogenesis in a 5-HT receptor and region specific manner
- Structural formation and functioning of the somatosensory system as well as the hippocampus
- Regulate the terminal development and ingrowth of dopamine

By the end of the first postnatal week there is already a rapid growth of serotonin dendrites into the form seen in the adult rat and by the third postnatal week the adult pattern is already well established (Murrin, Sanders & Bylund 2007). Serotonin levels in the central nervous system of the rat is generally low at birth but peak around PostND21-30 and then decline somewhat to adult levels (Whitaker-Azmitia 2001), these fluctuations in serotonin are mirrored closely in human children. In comparison with norepinephrine and dopamine, serotonin shows the least dramatic and most rapid development (Murrin, Sanders & Bylund 2007).

Alterations in serotonin during specific stages in development could have different behavioural outcomes, as prenatal serotonin administration in clinical and pre-clinical settings has resulted in anxiety-like behaviour in adulthood (Olivier et al. 2011). Changes have also been observed in social behaviour although reports of no behavioural alterations have also been documented (Olivier et al. 2011). Behavioural alterations seem to be dependent upon the type of SSRI as well as the route of administration (Kepser, Homberg 2015). Postnatal changes also seem to vary as changes in serotonin between PostND0-14 results in severe sensory impairments and deficits in motor coordination. Changes between PostND7-20 can lead to functional changes as well as changes in behaviour that seems to be autism-like in later-life (Andersen, Navalta 2004). Furthermore other studies have also

found early-life increases in serotonin to result in abnormal brain structure and function, sensory impairments and motor coordination deficits as well as behavioural abnormalities such as depressive and anxiety-like behaviour in adulthood (Kepser, Homberg 2015, Olivier et al. 2011). Findings in clinical studies seem to be similar to findings in rodents as SSRIs during pregnancy and lactation could modestly increase the risk for autism spectrum disorders in the child (Kepser, Homberg 2015, Croen et al. 2011).

The earlier maturation rates of the serotonergic pathways compared to that of the noradrenergic and dopaminergic pathway suggests that drugs affecting the serotonin system could most likely have more adverse effects during earlier stages of development. This is not hard to conceive when taking into account the key role serotonin plays in the regulation of neurodevelopment (Murrin, Sanders & Bylund 2007). However, it is evident that most studies focus on early-life treatment before or until PostND21 and then from adolescence (>PostND35) onwards, with very few studies focussing on the pre-pubertal period between PostND21-34. Therefore, a lot of research still needs to be done to determine the effect of early-life pharmacological interventions in animals and animal models of depression. Data suggest that even environmental enrichment during adolescence may reduce the heightened hormonal stress reactivity and impaired cognitive function in adulthood resulting from neonatal stress of maternal separation (Brenes, Rodríguez & Fornaguera 2008). Specifically, studies have shown that maternally separated rats exposed to enriched environments (e.g., larger housing, toys, and running wheel) during puberty, show reduced stress reactivity and greater cognitive abilities compared to their maternally separated counterparts that were exposed to standard laboratory environments (Brenes, Rodríguez & Fornaguera 2008). Similar results have also been obtained using enrichment during adolescence to offset negative physiological and behavioural aspects induced by prenatal stress (Eiland, Romeo 2013). These data not only demonstrate the importance of early-life adversity, but also suggest that specific interventions may alleviate these effects of adversity.

2.8 Synopsis

The neurobiological basis of depression and antidepressant action has been investigated extensively, but with an array of key questions remaining. Depression and anxiety disorders not only affect adults, but are frequently seen in children and adolescents. Fluoxetine, and more recently also escitalopram, are the only antidepressants approved for treatment of juveniles. Pre-clinical and clinical observations suggest that children and adolescents respond differently to antidepressants and that the SSRIs are more effective than other classes in this group, suggesting also a different neurobiology to that of adults. In this regard, few studies have investigated the effect of antidepressants on neurodevelopment, and it is uncertain whether antidepressant treatment early in life will have a positive or negative long-term outcome. Most work in this regard has been done in animals, rendering conflicting results. Discrepancies in results were mostly due to subtle neurobiological and

behavioural differences between animal species (rats versus mice) and strains (FSL, Sprague Dawley and Wistar), as well as differences between sexes, age groups, drugs used and drug dosages implemented. This emphasizes the need for a translational animal model with enhanced validity, and in particular a juvenile model with genetic predisposition to develop depressive-like behaviour.

Secondly, exercise has been suggested as an intervention with potential to improve mental health, also in children. A few studies also suggest that low and moderate intensity exercise may have positive effects and could be used as augmentative therapy to improve neuroplasticity as described above. In particular also, the induction of BDNF expression by physical activity plus pharmacological treatment combinations could represent an important intervention for further study, particularly in the treatment and management of juvenile MDD (Russo-Neustadt et al. 2001).

The current study therefore aimed to shed light on several research questions as derived from the literature. Firstly, it was investigated whether pre-pubertal treatment with fluoxetine and/or exercise will influence produce early effects (PostND35) and also whether and how it affects neurodevelopment and therefore affect behaviour of the FSL rat in early adulthood on PostND60. Furthermore it was investigated whether these pre-pubertal insults lead to changes in markers of oxidative stress, BDNF and corticosterone levels, and in addition whether these changes in biomarkers can explain the behavioural results obtained.

CHAPTER 3

RESEARCH ARTICLE

The current dissertation is presented in "article format" as recognized by the North-West University. The essential data has been compiled and presented as a research article prepared for submission to Behavioural Brain Research, an appropriate peer-reviewed scientific journal.

The current chapter was prepared according to the instructions to the author for this journal, as presented in Addendum C. The heading, table and figure numbers for this chapter will be done in accordance to that of the dissertation. The figures and tables will be presented in the text in accordance with the dissertation to benefit the reader. The results and discussion will be discussed under one heading in order to benefit the reader of this dissertation and not as two separate headings as normally required by the journal. However this will be changed for submission for publication. The references for this chapter will be found at the end of the article manuscript. The references for the dissertation as a whole will therefore be presented at the end of the document (see References).

The guidelines for the preparation of the article manuscript are outlined on the journal website: http:/www.journals.elsevier.com/behavioural-brain-research/, under "Guidelines for Authors".

The manuscript title, contributing authors and affiliations will appear on the next page. The abstract, Highlights and Keywords will also be presented on a single page. The main body of the manuscript follows the following structure: Introduction, Materials and Methods, Results and Discussion, Conclusions, Acknowledgements, References, Legends to Tables, Legends to Figures. However as mentioned earlier to benefit the reader all figures and tables have been included in the text and not at the end of the manuscript as normally required by the journal.

J.C. Schoeman conducted the behavioural and Neurochemical experiments, did the initial data work-up and statistical analyses, and wrote the first draft of the manuscript. C.B. Brink designed and supervised the study and assisted in the interpretation of the study data, as well as finalized the manuscript for publication. B.H. Harvey advised on the study design and proofread the final manuscript.

Title of article

Early-life exposure to fluoxetine and/or exercise on bio-behavioural markers of depression in early adulthood in stress sensitive rats.

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Highlights

- In FSL rats maximal exercise intensityincreases with age and should be adapted accordingly.
- Pre-pubertal fluoxetine and low intensity exercise alone exerts lasting antidepressant-like effects in early adulthood.
- The combination of pre-pubertal fluoxetine and low intensity exercise does not exert lasting antidepressant-like effects.

Abstract

Major depressive disorder in children is of great concern world-wide with only fluoxetine and escitalopram approved for treatment. Delayed onset of action, low remission rates, high rates of relapse and potential long-lasting consequences further complicates treatment and highlights the need for new treatment options. Several studies reporting on long-lasting effects of early-life treatment reported conflicting results, which may in part be explained by differences in the age of insult. The pre-adolescent period has mostly been overlooked, which may be an important shortcoming of current data. The anti-depressive effect of low intensity exercise, as a possible treatment option or antidepressant augmentation strategy, has also been found to be dependent on age and exercise intensity. We investigated in stress sensitive Flinder's Sensitive Line rats the lasting effect of pre-pubertal (i.e. postnatal day 21 (PostND21) to PostND34) fluoxetine and/or exercise on behaviour and neurobiological markers of depression, as observed long after treatment in early adulthood (PostND60 to PostND61). Pilot studies suggested that incrementally increased low intensity exercise or 5 mg/kg/day fluoxetine yield best results early after treatment. Furthermore, the data indicated that treatment with fluoxetine (5 mg/kg/day) or low intensity exercise during pre-adolescence exerts lasting anti-depressive like effects into adulthood, whereas the combination of fluoxetine plus exercise did not. In conclusion, the data suggest that optimal lasting effects of early-life interventions may require individualisation of antidepressant dose and/or exercise intensity according to the developmental age.

Keywords

Neurodevelopment, pre-puberty, fluoxetine, exercise, Flinders Sensitive Line rat

3.1 Introduction

Major depressive disorder (MDD) is one of the most challenging mental health problems of our time affecting an estimated 350 million people worldwide, at any given point in time [1]. Children are also affected and due to increased awareness and a rise in the number of juveniles diagnosed with MDD, it has become the most common mental health disorder in this age group [1]. In fact, MDD has an estimated prevalence of 2-5 % in children, associated with a fourfold enhanced risk of enduring or reoccurring in adulthood [2]. Additionally, severe depression often leads to suicide [3] and therefore it is not surprising that it is the fourth leading cause of death in pre-adolescent children [4]. An increase in the prescription rate of antidepressants in this age group has also been documented [1], highlighting the need for safe and effective treatment options in this age group.

During pre-adolescence the serotonin pathway is fully matured, whereas the noradrenergic and dopaminergic pathways are still developing, so that drugs that modulate serotonergic neurotransmission are more likely to be effective than those modulating other systems [5]. That said, current treatment options are limited to fluoxetine and escitalopram, both two selective serotonin reuptake inhibitors (SSRIs), as approved by the United States' Food and Drug Administration (FDA) for the use in children [6] older than 8 and 12 years respectively [7]. Other antidepressants, such as tricyclic antidepressants and even other SSRIs, have been shown to be ineffective in children [5,8]. In addition, a blackbox warning was issued by the FDA in September 2004, due to an increased risk of suicidal ideation in juveniles treated with SSRIs [9]. Lastly, the potential long-term consequences of early-life treatment in the developing brain have become a great concern in recent years. This has made prescribing a daunting task as prescribers have to weigh the risks and benefits of early-life treatment with the unknown risk of late-life consequences.

A few studies focused on the potential long-term consequences of early-life adverse events including stimulant and anti-depressant insults, in an attempt to shed some light on how these stimuli during the complex process of brain development could alter the brain's functional integrity in adulthood [10]. Neurodevelopment during pre-adolescent years presents a window when insults can induce permanent neurodevelopmental alterations [11]. Some pre-clinical data suggest negative outcomes of early-life treatment (PostND0-21) with SSRIs, such as arrested development of spine density [12], decreased density of the serotonin transporters [13], reduced bodyweight, reduced sexual functioning and/or increased anxiety [14], increased immobility in the forced swim test (FST) (depressive-like behaviour) [15] and decreased locomotor activity in the open field test (OFT) [16]. However, it has been suggested that an insult during the right developmental period could alter the course of development to exert beneficial lasting effects [11]. This idea is not farfetched and it has been demonstrated that fluoxetine treatment in rodents during adolescence (PostND35) may produce

decreased immobility in the FST and positively influence responsiveness to rewarding and aversive stimuli in adulthood [17].

Importantly, several methodological and other differences exist, which may explain contradictory research findings. Most animal studies employ healthy rodents, without any genetic predisposition to display depressive-like behaviour, consequently limiting the translational value of the findings. Secondly, pre-pubertal rodent neurodevelopment (PostND21-34) can be translated to that of human child (4 – 12 years), when antidepressant treatment is often indicated.

Although the pre-adolescent period might be more beneficial to exert enduring effects, antidepressant treatment is associated with bothersome side-effects, a delayed onset of action [17], low remission rates and a high rate of relapse following discontinuation [18], highlighting the need for new treatment modalities. Such interventions typically include psychotherapy, life-style adjustments and support groups.

Exercise is another suggested treatment option with significant efficacy demonstrated in adults [19,20,21], children [22] and rodents [23], although data on children are limited. Exercise has also been proposed as an augmentative strategy to antidepressant treatment, due to the putative synergistic effects with antidepressants as well as the advantage of being a relatively safe and a low cost intervention. The antidepressant effects of exercise have been suggested to result from increased levels of monoamines, neurotrophins, anti-inflammatory markers and anti-oxidants [21,24,25]. Immediate effects of exercise seem to be dependent upon age as well as intensity [10,26]. In a study conducted by de Almeida et al., (2013) it was found that low intensity exercise during pre-adolescence exerts beneficial effects, whereas high intensity exercise results in negative effects [26]. However, high intensity exercise appears to be more beneficial during adolescence [26], adding further support to the idea that treatment could have differential effects depending on the time of treatment initiation during development. Nevertheless, few studies have explored the potential lasting effects of exercise as a treatment option for depression.

In the current study we investigated whether pre-adolescence presents a window of opportunity, particularly in genetically susceptible rats, to exert beneficial lasting effects on behavioural and biomarkers of depression. We also explored whether the combination of fluoxetine and exercise will yield an augmentative long-lasting anti-depressant-like effect in adulthood compared to either two treatment options alone.

3.2 Materials and Methods

3.2.1 Animals

Male Flinders Sensitive Line (FSL; n = 167) and Flinders Resistant Line (FRL; n = 12) rats were bred, supplied, and housed at the Vivarium at the Pre-Clinical Drug Development Platform (PCDDP) of the North-West University. The original rat colonies were obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA. The FSL rat is a validated genetic animal model of depression, displaying face, construct and predictive validity, whereas the FRL rat serves as a model control [27,28]. We also demonstrated in the current study under our experimental conditions that FSL rats display enhanced immobility in the forced swim test (FST) , as compared to the FRL rat (192.8 ± 4.010 vs 151.9 ± 5.296 seconds per 5 minutes; $p < 0.0001$). The study aimed to employ 16 rats per group, depending on the required sample size estimated for the particular test. However, smaller numbers were employed in some groups due to lower birth rates of male pups, a few non-running animals that were excluded [29], and two animals that were removed from the study due to foot injuries on the treadmill. Animal numbers per groups are indicated in the results section.

The rats were housed in groups of three per cage in cages with corncob bedding changed weekly and the environmental temperature maintained at 22 ± 1°C and a relative humidity of 50%. A 12h light/dark cycle (lights on at 06:00 and off at 18:00) was followed and food and water were available *ad* libitum. Pups were weaned on PostND21 and body weight was measured daily during treatment from PostND21 until PostND34. From PostND35 to PostND60 rats were housed under normal conditions, without any treatment. Animal wellbeing was routinely monitored on specially developed monitoring sheets throughout the study, as well as specifically monitored during and after each injection and exercise session. In addition, animals were handled daily by the researcher from PostND16 onwards to familiarise the animals to human handling.

All experiments conformed to the guidelines of the South African National Standards: The care and use of animals for scientific purposes (SANS 10386:2008) and were approved in accordance with the regulations set by the animal research ethics committee (AnimCare) of the North-West University (ethics approval no. **NWU-00148-14-A5**).

3.2.2 Drug treatment

Animals received via subcutaneous (s.c.) administration either saline (vehicle) or fluoxetine (a kind gift from Jade Pharmaceuticals, South Africa; Fluoxetine HCl USP, Batch nr: FX/1505001) once daily between 7:00 and 10:00 from PostND21 to PostND34. Fluoxetine for injection was freshly prepared daily by dissolving crude fluoxetine powder in 0.9% saline and administered in a volume of 2ml/kg [30]. Such 14 day treatment period is generally considered sub chronic and longer than the minimum required to produce antidepressant-like effects in FSL rats [31]. The s.c. route of

administration has a predictable bioavailability, comparable to that of an intraperitoneal injection, but with less injection stress to the animals, particularly in young rats. Fluoxetine had been successfully delivered with s.c. administration before [32]. We administered fluoxetine HCl at an indicated dose of either 5 or 10 mg/kg/day as has been found to be effective in literature [33]. The period between PostND21 to PostND34 represents pre-puberty in rodents when the serotonergic pathway is already matured, while the noradrenergic and dopaminergic pathways are still developing [5]. After treatment rats were either submitted to behavioural analyses on the following day (PostND35) or left in normal housing conditions during a period of 26 days of drug washout, until behavioural analyses on PostND60 (early adulthood) and brain dissection and trunk blood collection for neurochemical and peripheral testing on PostND61.

3.2.3 Exercise

3.2.3.1 Treadmill Familiarization

Prior to the commencement of exercise, all animals were familiarised to the treadmill (custom-built by the Dept. Instrument Making, Potchefstroom Campus, North-West University) from PostND16 to PostND20, i.e. five consecutive days [34,35,36] of comfortable walking for 10 minutes/day [37].

3.2.3.2 Reinforcement

Rats were motivated to run on the treadmill by means of negative reinforcement [29] with a shocking grid at the back of the treadmill, yielding sufficient electrical shock (1mA, 3Hz) to be uncomfortable but not painful or harmful. Importantly, during familiarisation and the chronic exercise regimen animals that stopped running even after reinforcement were removed from the treadmill after five seconds and used as control rats [29]. We observed a small number of rats (< 10%) who display "non-runner" behaviour, which is in line with previous reports [29]. During the exhaustion test, whenever a rat touched the grid four times within a period of 1 minute, it was considered indicative of exhaustion and the rat was immediately removed from the treadmill.

3.2.3.3 Exhaustion Test

Animals were familiarized to the treadmill as described above. Thereafter, animals (n=36) were divided into two groups (n=18 each) and submitted to alternate day exhaustion tests performed at commencement of the rodent wake cycle 18:00 - 22:00, with one group on PostND21, 26 and 32 and the other on PostND23, 28 and 34. Thereby animals were allowed to rest on the day following the exhaustion test, yet were subjected to further familiarization on all other days.

For the exhaustion testing rats were subjected to treadmill running to determine the maximum speed (intensity) at which the FSL rats can run, similarly to what was described before [38,39,40,41,42]. Each rat started at a low initial speed (2.5 m/min) which was increased with 2.5 m/min every 3

minutes until the point of exhaustion (i.e. failure of the rat to continue running). The time to fatigue (in minutes) and workload (in m/min) were taken as indexes of exercise capacity, which in turn were taken as indirect estimates of VO_2max. This procedure was used to establish whether VO_2max increases as rats developed from PostND21 to PostND34, and in particular to determine the eventual treadmill speed required at a given age to reach the indicated percentage of VO_2max in following experiments.

After determining the maximum intensity (VO_2max = 100%) at which FSL rats can run over a period of 20 minutes, using the maximal distance covered (time to exhaustion x speed at point of exhaustion) and dividing it by 20 minutes (exercise period), high (85%), moderate (70%) [43] and low (55%) [44] intensities were calculated. The intensities, expressed as a percentage of VO_2max in animals [43], correlate with human athlete percentages of VO_2max for low and moderate intensity exercise [45,46].

3.2.3.4 Chronic Exercise Regimen

Before the start of the exhaustion test animals were familiarized to the treadmill as described above. Animals were subjected to sedentary (zero speed), low, moderate and high intensity treadmill exercise once daily (18:00-22:00) for 30 minutes from PostND21 to PostND34. Low intensity, moderate as well as high intensity exercise was used as determined in the previous section. High intensity exercise was, however, immediately suspended upon observation of foot injuries and the incidents reported according to guidelines.

Sedentary animals were taken out of their cages and placed on a still-standing treadmill for 30 minutes during the time other rats underwent treadmill running. During the 30 minutes of daily training, the speed was increased every five (5) minutes for the first two (2) sets of five (5) minutes (warm-up) where after it was increased a final time for the last twenty (20) minutes of the training session [47]. The first 5 minutes were at a comfortable walking speed, being 33% of the intended exercise intensity, where after the intensity was increased to 67% of the intended intensity for 5 minutes and then to 100% of the intended speed for the final 20 minutes of the 30 minute session [37,47,48,49,50]. After treatment rats were either submitted to behavioural analyses on the following day (PostND35) or left in normal housing conditions during a period of 26 days of washout, until behavioural analyses on PostND60 (early adulthood) and brain dissection and trunk blood collection for neurochemical and peripheral testing on PostND61.

3.2.4 Behavioural Analyses

After the 14-day period of treatment (vehicle/fluoxetine, sedentary/exercise or drug plus exercise), animals were subjected to the least stressful open field test (locomotor activity and anxiety) followed by the more stressful FST. We demonstrated before, that under our experimental conditions, foregoing tests do not affect the outcome of the subsequent consecutive tests if they are ordered from

least to most stressful [51]. The behavioural tests were performed for all treatment groups either early after the pre-puberty intervention period (i.e. injection and /or exercise) on PostND35, or later in life after drug wash-out (withdrawal) and normal housing on PostND60, representing early adulthood. Testing commenced one hour after the start of the dark cycle (i.e. 19:00) in order to ensure normal initial foraging and activity of nocturnal animals. Tests were carefully spaced to allow 30 minutes between each test in order for animals to habituate to the environment.

3.2.4.1 Open Field Test

The Open Field Test is a test used for measuring locomotor activity, which is a parameter used to test the general ability of the animal to move and negotiate its surrounding.

The apparatus used for the test consisted of a 1 m^2 square test arena, digitally marked with sixteen 25 × 25 cm smaller squares, and the arena surrounded by opaque black, vertical walls [52]. On the day of testing following foraging and habituation (see above), each rat was placed in the centre of the arena and allowed to explore the environment for 5 minutes under red light of 80 lux intensity [52]. During this time the rat was video-taped by a camera mounted above the test arena and afterwards scored using Ethovision XT 11 software (Noldus Information Technology BV, Wageningen, Netherlands). The total number of lines crossed during the session was used as a measure of general activity, whereas the number of entries into the central square of the arena provided a measure of anxiety-like behaviour. Rats have a natural inquisitive nature, prompting them to investigate spaces, yet an aversion towards illuminated open spaces and thus will tend to spend less time in the centre of the test arena (i.e. closer to the protective walls) when anxiety levels increase. Animals that spent more time close to the arena walls were regarded as displaying more anxiety-like behaviour [53].

3.2.4.2 Forced Swim Test

The FST was used to assess depressive-like behaviour in rodents [54]. Lucki [55] adjusted the FST to distinguish serotonergic and noradrenergic mechanisms [33]. The FSL rat represents a well described validated animal model of depression that displays depressive-like behaviour (enhanced immobility) without the pre-conditioning swim trial 24 hours prior to the testing swim trial [27].

As previously described by Steyn et al., 2011 [56] the apparatus consists of four cylindrical tanks, each with dimensions 40 cm high and 20 cm in diameter, and spaced next to one another. Each tank was filled to a depth of 30 cm with water, maintained at 25 ± 1^{o}C. The test was performed during the dark cycle under red light. On the day of testing following foraging and acclimatisation, each rat was placed in the cylinder and allowed to swim for 7 minutes, whilst behaviour was recorded by a camera mounted with frontal view of the cylinders. From the video recording behaviour was scored from the mid 5 minutes of the recorded swimming by an experimenter blind to the test group.

Behaviour scored included immobility, climbing and swimming, as previously described [33,52,57]. Enhanced adrenergic and dopaminergic neurotransmission are associated with increased climbing, whereas enhanced serotonergic neurotransmission is associated with increased swimming behaviour [57].

- Immobility is defined as no active movements 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.

3.2.5 Statistical Analyses

When comparing only two data points, the Student's t test was used. Normality of the data was determined using the Shapiro-Wilk test. For multiple comparisons of data a non-parametric two-way ANOVA based on ranked data was performed if the assumption of normality was violated. In other instances, the two-way ANOVA was used, and when this analysis indicated interaction between the main factors (i.e. drug and exercise treatment, respectively), it was followed by the Dunnett or Tukey posthoc test depending on the purpose of analysis. The Dunnett posthoc test was used when the mean of all treatment groups were compared to that of the control group, whereas the Tukey posthoc test was used for multiple comparison of all groups. Three-way analyses were not necessary, since the third main factor, namely age at PostND35 vs PostND60, were not directly compared. GraphPad Prism® version 6 (GraphPad Software, San Diego California USA, www.graphpad.com) was used for statistical analysis and graphical presentations, except for non-parametric statistical analyses when indicated, where IBM SPSS Statistics version 22 was used (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). A 5% confidence limit for error was taken as statistically significant ($P < 0.05$).

3.3 Results and discussion

Figure 3.1 represents the relationship between maximal treadmill speed and postnatal age in FSL rats.

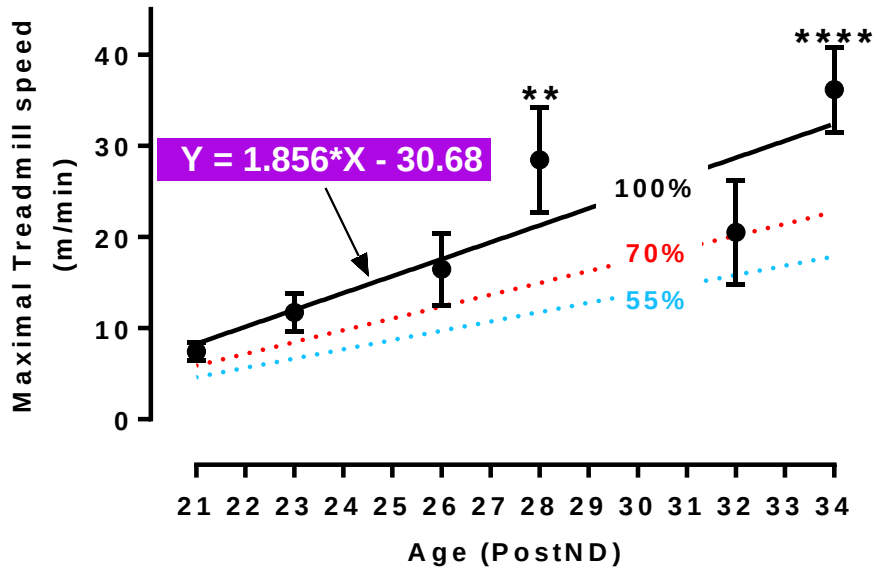

Figure 3-1: Indirect VO_2max across pre-pubertal development in FSL rats.

Increase in maximal exercise intensity over time, represented by maximal treadmill speed in m/min from PostND21 to PostND34 in FSL rats, n= ± 16 rats per group, as non-runners were excluded. Data points are mean ± SEM and the line represent the correlation between speed and age, defined by the equation y = 1.855x – 30.58, with R^2 = 0.7642 (Pearson r correlation). PostND = postnatal day. ** $p < 0.01$; **** $p < 0.0001$.

It can be seen in Figure 3-1 that the maximal exercise capacity of pre-adolescent FSL rats increases gradually with age from PostND21 to PostND34. The linear relationship between age and maximal speed is described by y = 1.855x – 30.58 (R^2 = 0.7642). The one-way ANOVA indicates that differences in maximal speed at different ages are statistically significantly (F [5,84] = 6.613, $p < 0.0001$), with the Dunnett's multiple comparison test indicating a significant increase in the maximal speed on PostND 28 (PostND21 vs PostND28; $p < 0.01$) and on PostND34 (PostND21 vs PostND34; $p < 0.0001$).

The data in Figure 3-1 therefore demonstrates that maximal exercise intensity (VO_2 max) increases with age from PostND21 to PostND34. The linear correlation between maximal treadmill speed (m/min) and age (PostND) is significant, and the conclusion is also supported by a statistically significant increase in maximal intensity from PostND21 until PostND34. It can be deduced that exercise intensity (VO_2max) increases in a linearly related manner across pre-pubertal development of the FSL rat, as described mathematically by the equation provided. This finding highlights the need to adapt the targeted exercise intensity according to age, rather than keeping it constant throughout

treatment as done in previously reported studies [26,47,58,59]. To our knowledge this is the first time that an age-related exercise regimen was developed in pre-pubertal rodents.

Figure 3-2 represents behavioural data of pilot studies as observed in the forced swim test (FST) and open field test (OFT) on PostND35 in FSL rats.

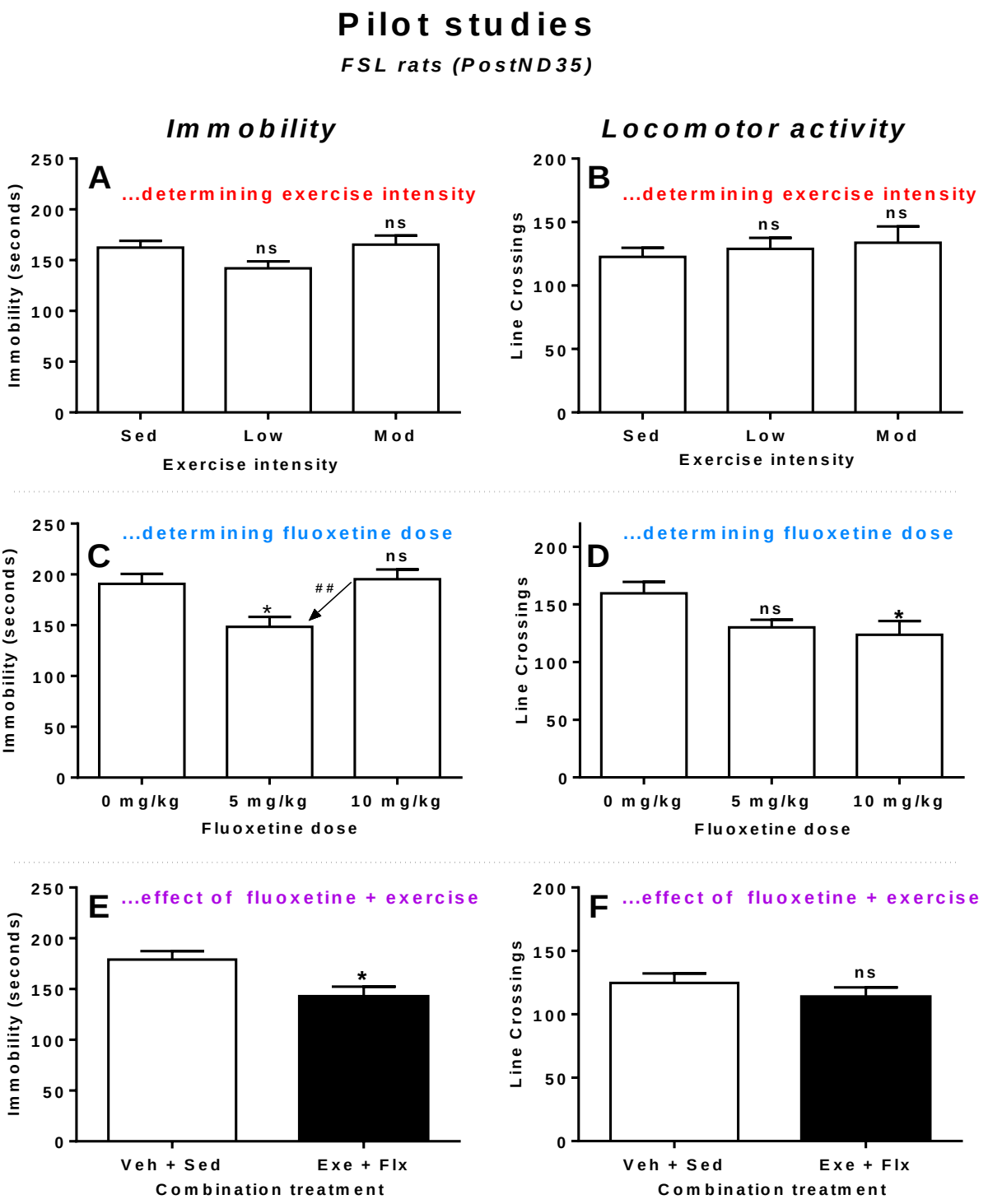

Figure 3-2: Pilot studies on behaviour of FSL rats on PostND35

(A) Immobility in the FST on PostND35 following treatment with low (n=15) and moderate (n=16)

intensity exercise compared to the sedentary control (n=17). (B) Number of line crossings in the OFT after treatment with low and moderate intensity exercise when compared to a sedentary control. (C) Immobility on PostND35 after fluoxetine treatment at dosages of 5 mg/kg/day (n=7) or 10 mg/kg/day (n=7) compared to the vehicle control (n=8). (D) Number of line crossings on PostND35 in the OFT after fluoxetine (n=13) and saline treatment (n=8). (E) Immobility on PostND35 after fluoxetine (5 mg/kg/day) treatment plus low intensity exercise (n = 12) compared to the vehicle plus sedentary control group (n = 12). Data points represent the mean ±SEM. Statistical analyses are reported in the text, with ns = not significantly vs control; * $p < 0.05$ vs control; ## $P < 0.01$ vs indicated test group. Sed – sedentary, Low = low intensity exercise (55% VO_2max) and Med = medium intensity exercise (70% VO_2max), Veh = vehicle saline control, Flx = fluoxetine 5 mg/kg, Exe = exercise at low intensity.

The Shapiro-Wilks test as an analysis of variance for normality indicated that the assumption of normality was true for all data sets in Figure 3-2 ($p > 0.05$ in all cases), so that the ordinary one-way ANOVA and Student's t test could be applied as appropriate.

In Figure 3-2 A & B an ordinary one-way ANOVA of the data indicated no statistically significant differences between the exercise-treated and the sedentary control rats in both Figure 3-2A immobility in the FST ($F [2, 39] = 2.858$, $p = 0.0695$) and Figure 3-2B number of line crossings in the OFT ($F [2, 39] = 3.3685$, $p = 0.6942$).

In Figure 3-2C an ordinary one-way ANOVA of the data ($F [2, 19] = 6.911$, $p = 0.0056$) indicated a statistically significant difference regarding immobility between treatment groups on PostND35. Tukey's post hoc analyses indicated that fluoxetine 5 mg/kg/day significantly decreased immobility in the FST when compared to both the vehicle control ($p < 0.05$). This effect was reversed when using fluoxetine 10 mg/kg/day, with immobility not significantly different from vehicle control and statistically significantly enhanced relative to the effect observed with 5 mg/kg/day ($p < 0.01$). In Figure 3-2D an ordinary one-way ANOVA of the data ($F [2, 19] = 3.931$, $p = 0.0373$) indicated statistically significant differences between treatment groups and the Tukey's post hoc analyses indicated that fluoxetine 10 mg/kg/day, but not 5 mg/kg/day, significantly decreased the number of line crossings when compared to the vehicle control ($p < 0.05$).

In Figure 3-2E the unpaired Students' t-test of the data (179.0 ± 8.295 vs 142.3 ± 10.12, $p = 0.0104$) indicated that the combination of fluoxetine 5 mg/kg/day with low intensity exercise significantly decreased immobility in the FST when compared to the vehicle plus sedentary control. As can be seen in Figure 3-2F no significant differences in number of line crossing were observed between these two treatment groups.

The data obtained from the FST (Figure 3-2A) indicate that sub chronic low and moderate intensity exercise in pre-pubertal FSL rats does not produce early antidepressant-like effects, as suggested by immobility on PostND35 (i.e. one day after the last day of treadmill exercise). Utilising another strain of rat, and also following maternal separation to induce depressive-like behaviour, others reported low intensity treadmill exercise from PostND21-30 to induce significant decrease in immobility scores in

the FST [59]. Several other reports also suggest a beneficial effects of low intensity exercise on physiological and psychological resilience, so that low intensity exercise, more so than moderate and high intensity exercise, is generally assumed to be effective in reducing depressive-like behaviour in rodents [26,47,58,59]. Furthermore, low intensity exercise has been proposed as augmentative strategy in the treatment of depression. Data on exercise in FSL rats, a genetic model of depression, are limited and most researchers employ voluntary wheel running as a form of exercise. In this regard, it was found that four weeks of voluntary running of FSL rats significantly decreased immobility as compared to the control animals [24], again supporting the idea that low intensity exercise may hold potential as an augmentation strategy.

In the current study we also observed no differences in locomotor activity of forced exercise versus sedentary control animals (Figure 3-2B), suggesting that locomotor activity was also not affected early on by exercise. This is of note, since we did not know whether enhanced fitness of rats would affect this parameter. The locomotor data also confirms that the lack of change in immobility is not due to blunting of behavioural effects by altered locomotor activity, but indeed represents a lack of altered psychomotor activity.

Interestingly we found fluoxetine administration in pre-pubertal rats are only effective to decrease immobility in the FST (Figure 3-2C) on PostND35 when administered at a low dose of 5 mg/kg/day, as opposed to no effect seen when administered at a higher dose of 10 mg/kg/day. Our finding is supported by previous findings that fluoxetine 5 mg/kg/day is sufficient to inhibit the serotonin transporter in juvenile rats [60,61]. Furthermore, the lower dose of fluoxetine also correlates with the clinical scenario [9,59], since rats generally receive doses of fluoxetine 10 times higher than humans due to increased liver metabolism. In this regard a typical dose of 20 mg/day in humans yield a dose of roughly 0.3 to 0.9 mg/kg/day [9], so that the corresponding rodent dose would be 3 to 9 mg/kg/day. The lower dose of 5 mg/kg/day for FSL rats would fall into this range. In addition, the higher dose of 10 mg/kg/day fluoxetine significantly decreased the number of line crossings in the OFT in the current study (Figure 3-2D), suggesting that altered locomotor activity in pre-pubertal FSL rats may blunt any psychomotor related decreases in immobility [33]. Previous studies also found that higher doses of fluoxetine affect motor function [62] further supporting the use of the lower dose of 5 mg/kg/day in pre-pubertal FSL rats for the main study.

Lastly, data obtained from (Figure 3-2E) indicate that animals treated with the combination of fluoxetine plus low intensity exercise does display significantly less immobility in the FST when compared to the vehicle plus sedentary control, suggesting that the augmentative strategy holds potential to explore as an effective treatment option in pre-pubertal FSL rats in the main study, investigating lasting effects. In the current study fluoxetine plus exercise did not change locomotor activity (Figure 3-2F), confirming that the observed effect in the FST is related to psychomotor alterations and not to locomotor activity. This finding is in line with several previous reports of the

beneficial effect of exercise as potential augmentation strategy with antidepressants in adult rodents [63,64], human adolescence [65] and adults [23,24].

Figure 3-3 represents lasting effects (PostND60) of pre-pubertal treatment on depressive-like behaviour and locomotor activity in FSL rats.

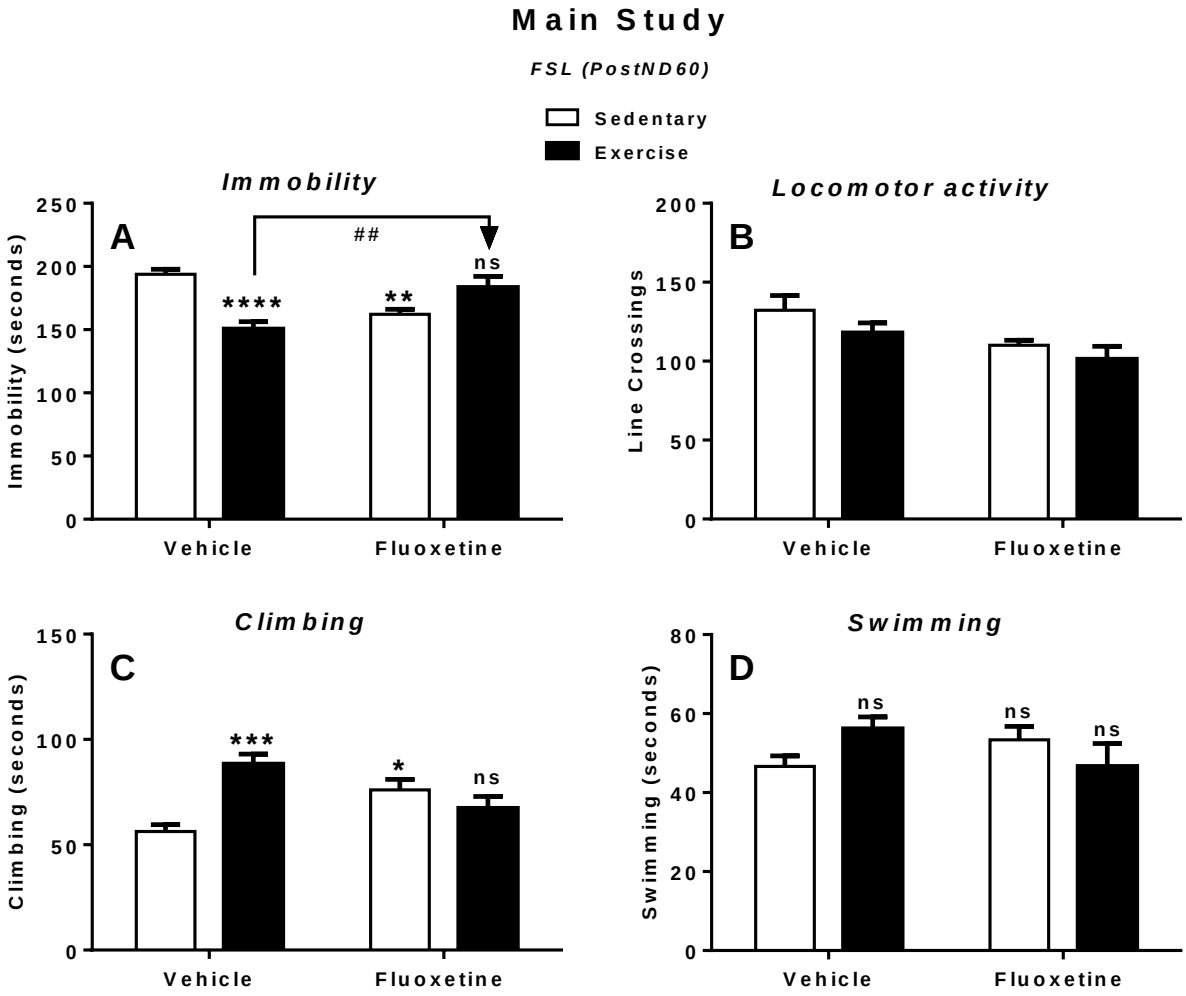

Figure 3-3: Behaviour of FSL rats on PostND60 after pre-pubertal treatment with low intensity exercise, fluoxetine 5 mg/kg/day and the augmentation of fluoxetine with exercise.

(A) Immobility in the FST on PostND60, after 26 days washout following pre-pubertal treatment with low intensity exercise (n = 12), fluoxetine 5 mg/kg/day (n = 12) and the augmentation of fluoxetine 5 mg/kg/day with low intensity exercise (n = 12) compared to the vehicle control (n=12). (B) Number of line crossings in the OFT on PostND60. (C) Climbing in the FST on PostND60. (D) Swimming on PostND60 in the FST. . Data points represent the mean ±SEM. Statistical analyses are reported in the text, with ns = non-significant vs control, * $p < 0.05$ vs control, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control, ## $p < 0.01$ vs indicated test group.

The Shapiro-Wilks test as an analysis of variance for normality indicated that the assumption of normality was true for all data sets in Figure 3-3 ($p > 0.05$ in all cases), so that the ordinary one-way ANOVA and Student's t test could be applied as appropriate.

In Figure 3-3A the two-way ANOVA of the data ($F [1,44] = 29.32$; $p < 0.0001$) indicated a statistically significant interaction between drug treatment and lifestyle intervention regarding immobility on PostND60. The Tukey post hoc analyses for multiple comparison indicated that low intensity exercise alone significantly decreased immobility in the FST when compared to the vehicle plus sedentary control group ($p<0.0001$). Administration of fluoxetine 5 mg/kg/day alone also reduced immobility when compared to the vehicle plus sedentary control group ($p<0.01$). However, when fluoxetine was added to exercise, the effect was reduced as compared to exercise alone ($p < 0.01$). The difference in immobility between fluoxetine alone and fluoxetine plus exercise did, however, not reach statistical significance in the FST.

In Figure 3-3B the two-way ANOVA ($F [1,44] = 0.1604$; $p = 0.6907$) of the data indicated that there was no statistically significant interaction between drug treatment and lifestyle therapy regarding the number of lines crossed in the open field test (OFT), hence no post hoc analysis was performed. However, the effect of fluoxetine was statistically significant ($F [1, 44]$ $p = 0.0095$).

In Figure 3-3C the two-way ANOVA of the data ($F [1, 44] = 18.68$; $p < 0.0001$) indicated a significant interaction between drug treatment and a lifestyle intervention regarding climbing behaviour in the FST. The Tukey post hoc analysis for multiple comparison indicated that low intensity exercise alone significantly increased climbing behaviour in the forced swim test (FST), when compared to the vehicle plus sedentary control group ($p<0.001$). Administration of fluoxetine 5 mg/kg/day alone also increased climbing behaviour when compared to the vehicle plus sedentary control group ($p<0.05$). However, the climbing behaviour of the fluoxetine plus exercise groups was not statistically significantly different from the vehicle plus sedentary group.

In Figure 3-3D the two-way ANOVA ($F [1, 44] = 4.316$; $p = 0.0436$) indicated a significant interaction between drug treatment and lifestyle intervention regarding swimming behaviour in the FST. However, the Tukey post hoc analyses indicated no significant differences in swimming behaviour between in any of the treatment groups.

The data obtained from immobility in the FST (Figure 3-3A) on PostND60 in FSL rats demonstrate that pre-pubertal exposure to low intensity exercise alone exerts long-lasting anti-depressive-like effects into early adulthood, following 26 days of normal housing and no exercise, as compared to the vehicle plus sedentary control group. The difference in immobility is attributed to enhanced adrenergic and/or dopaminergic (catecholaminergic) activity as increased climbing behaviour (Figure 3-2C) [66], with no differences in swimming behaviour (Figure 3-2D) observed. This was not expected, since it is widely accepted that the antidepressant-like effects of exercise is predominantly

due to enhanced serotonin neurotransmission [23,24]. This is of note, since the data suggest that pre-pubertal exercise may in fact affect neurodevelopment in such a manner that it has long-lasting effects on noradrenergic and/or dopaminergic neurotransmission. The ANOVA analysis of the data in Figure 3-3B also indicated that exercise did not affect locomotor activity, suggesting that the observed reduction in immobility relates to psychomotor activity and not to locomotor activity. To our knowledge, this is the first report of long-lasting beneficial effects into adulthood of treadmill exercise during pre-pubertal development in FSL rats.

Similar to the above mentioned findings we observed pre-pubertal FSL rats treated with sub-chronic fluoxetine to display decreased immobility in the FST in early adulthood, following a 26 day washout period, as compared to the vehicle plus sedentary control group. The decreased immobility is attributed to enhanced noradrenergic and/or dopaminergic activity as observed in increased climbing behaviour (Figure 3-3C) with no changes in swimming behaviour (Figure 3-3D) in the FST [33]. This is similar to what was seen with the exercise alone group described above. This was not expected, since fluoxetine as an SSRI is expected to enhance serotonergic neurotransmission [67], and hence swimming behaviour. As with exercise, the data suggest that pre-pubertal fluoxetine may affect neurodevelopment in such a manner that it has long-lasting effects on noradrenergic and/or dopaminergic neurotransmission. The ANOVA analysis of the data in Figure 3-3B also indicated that fluoxetine reduced locomotor activity. This was most prominent in the fluoxetine plus exercise group, suggesting that locomotor activity could potentially have blunted any antidepressant-like behaviour (reduced immobility) in the fluoxetine plus exercise group (Figure 3-3A). A previous study in our laboratory reported increased basal levels of dopamine in early adulthood of FSL rats exposed to pre-pubertal fluoxetine treatment when compared to vehicle treated rats [68], which may, at least in part, explain the increase in climbing behaviour observed in the current study. Interestingly, research on lasting effects of pre-pubertal exposure have mostly been overlooked with most research focussing on either early-life (<PostND21) or the adolescent period (>PostND35). Adolescent fluoxetine treatment demonstrated results comparable to that of the current study, such as decreased immobility in the FST [17,69], as well as enhanced reward processes, linked to dopamine [17]. These findings indicate that increases in serotonin specifically during the pre-pubertal period could potentially cause alterations in monoaminergic systems to such an extent that particularly dopamine is increased resulting in lasting antidepressant-like effects in early adulthood (PostND60).

In contrast to the above, the combination of fluoxetine plus exercise did not affect behaviour in the FST (Figure 3-3) on PostND60 compared to the vehicle plus sedentary control, a finding contradicting our working hypothesis. As mentioned above the pre-pubertal period is often overlooked, but interestingly similar findings such as increased immobility in the FST and reduced exploratory behaviour have also been reported with early-life (PostND0-21) increases in serotonin [16], as would be expected from fluoxetine and exercise treatment [23,24,33,57]. In addition increased anxiety,

reduced aggression, increased REM sleep and anhedonia has also been reported, although positive outcomes such as decreased impulsivity and improved learning and memory have also been observed [16]. The combination treatment displayed the most prominent decrease, although not significant, in locomotor activity (Fig 3-3B). Decreased locomotor activity has been correlated to decreased levels of dopamine [70] a relevant observation when taking into account that early-life (PostND0-21) increases in serotonin [71] leads to reduced dopamine levels, associated with decreased aggressiveness in adulthood [71].

The early maturation of the serotonergic pathway (PostND21) in rats compared to that of the noradrenergic and dopaminergic pathways [5] have mostly been the culprit for the paradoxical outcomes observed in early-life and adolescent or adulthood treatment with SSRIs. The findings observed in the current study therefore shed light on paradoxical outcomes observed within a single developmental period between early-life and adolescence. These findings highlight the importance of the pre-pubertal period as a period during development that is particularly sensitive to insults and more specifically alterations in serotonin concentration. The integral role serotonin plays in neurodevelopment as well as serotonin reaching peak levels during this period (PostND21-30) [72] further supports the importance of the pre-pubertal period and the role of serotonin. Pre-pubertal exposure to treatment strategies affecting serotonin can influence the development and maturation of other monoamine systems [5]. Interestingly the influence on other monoaminergic systems seems to be dependent upon the degree of serotonin alteration, as seen in the differential outcomes between exercise, fluoxetine and the combination therapy. In the current study, we propose that the higher elevation of serotonin levels (i.e. by fluoxetine plus exercise) led to behavioural outcomes resembling that of very early-life treatment, whereas moderate elevation of serotonin levels (i.e. by fluoxetine or exercise alone) led to behaviour resembling that of adolescent treatment. This would be in line with the idea that the degree of serotonin modulation during the pre-pubertal period can lead to differential behavioural outcomes. This observation could also be explained by the "equal but opposite" hypothesis [12,73], postulating that the sensitized response of receptors to increased serotonin in early-life could lead to an opposite outcome i.e. desensitized receptor response in adulthood. Supporting this idea is that the combination of fluoxetine plus exercise yielded early antidepressant-like effects on PostND35 in the current study, yet this combination did not result in lasing antidepressant-like effects as observed on PostND60. In addition, early-life exercise or fluoxetine alone resulted in lasting antidepressant-like effects into early-adulthood, suggesting that the overstimulation of the combination leads to desensitisation. Lastly the effect of withdrawal could also have influenced results in the "higher dose" augmented group as withdrawal after chronic exposure to antidepressant and stimulants often lead to depressogenic effects [74].

These findings support the notion that the time of insult during development as well as the dosage of treatment results in differential behavioural outcomes in adulthood. It could therefore be deduced that

pre-pubertal exposure to combined therapy results in an immediate antidepressant-like effect, while long-lasting depressogenic effects occur after prolonged discontinuation of chronic exposure, putatively via effects on neurodevelopment and a resulting effect on regional brain monoamines [75].

3.4 Summary and Conclusion

Long-lasting effects of early-life treatment have become a great concern in recent years as several studies have found paradoxical outcomes in both neurology and behaviour in adulthood after early-life exposures and insults. However, differences in timing of insults could explain different outcomes in adulthood suggesting a sensitive period in neurodevelopment that can possibly lead to beneficial enduring effects [76]. Therefore we explored the pre-pubertal period as such a developmentally sensitive period. In rodents the serotonin system is fully matured by PostND21 (onset of pre-puberty), whereas the noradrenergic and dopaminergic systems only mature by PostND35 [5]. Therefore, exposure of the mature serotonergic system of juveniles to SSRIs may in fact augment serotonin-mediated behavioural effects [77], and also modulate serotonin-mediated neurodevelopmental effects, including its impact on the developing adrenergic and dopaminergic systems. However administration of SSRIs to an immature serotonergic system (PostND21 <), impacts the developing serotonergic, adrenergic and dopaminergic systems, resulting in significantly different outcomes in both early and later life. Hence by optimising serotonin concentration during pre-adolescence in genetically susceptible individuals, may potentially be manipulated to yield beneficial long-term effects.

The findings of the current study highlight the need to adapt exercise intensity according to age rather than keeping it constant in developing rodents. Furthermore the current study found that pre-pubertal low intensity exercise initially did not alter depressive-like behaviour early after treatment, in contrast to the initial antidepressant-like effects observed in animals treated with fluoxetine or the combination of fluoxetine and exercise. The antidepressant-like effects observed in the latter groups are expected to be due to enhanced serotonergic neurotransmission. Interestingly, following 26 days of no exercise and/or washout, we observed enhanced antidepressant-like effects in the exercise alone group as well as the fluoxetine alone group. Lasting enhanced climbing behaviour was observed in the FST in both of these groups in early adulthood, suggesting increased dopaminergic and/or noradrenergic neurotransmission [33,66], and which was not expected of treatment strategies predominantly affecting serotonergic neurotransmission. Augmentation of fluoxetine with exercise, however, did not exert long-lasting behavioural effects as we originally proposed, most likely as a result from a lasting decrease in dopaminergic neurotransmission. This conclusion can be drawn from observations that escape related behaviour in the FST was unchanged, whereas locomotor activity in the open field test was reduced.

These findings may be explained by modulation of monoamine neurotransmission following altered serotonin concentrations during early-life neurodevelopment. Taking into account the outcomes of the current study, we propose that a lower dose of fluoxetine alone or low intensity exercise alone supports serotonin concentration in genetically susceptible rodents, resulting in beneficial effects in early-life, lasting into adulthood. The combination of fluoxetine plus exercise exerts a decrease in depressive-like behaviour early after treatment, but not lasting into adulthood, putatively related to resulting monoaminergic deficits in adulthood. Furthermore, the current study supports the idea that the pre-adolescent period presents a sensitive period in neurodevelopment when antidepressant treatment can have lasting effects into adulthood, and that such lasting effects may be dependent on both drug dosage and exercise intensity. Further investigations are needed in order to elucidate the mechanism behind these results.

Prospective studies should investigate long-lasting neurobiological effects of pre-pubertal fluoxetine and exercise, in particular its modulating effects on dopaminergic, serotonergic and noradrenergic neurotransmission, in order to elucidate the differential effects observed. Taken together, the current study suggests that early-life treatment should be individualised and adapted according to the neurodevelopmental age of the individual.

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CHAPTER 4

SUMMARY, CONCLUSION AND RECOMMENDATIONS

In order to provide a comprehensive overview of this study as a whole, the results and discussion (Chapter 3 and Addendum A) will be summarized and further discussed within this chapter (Chapter 4). This chapter will take into account the objectives of this study and ultimately culminating in a final conclusion and recommendations for future studies.

4.1 Summary of results

The current study consisted of 5 phases (see Chapter 1) that were done in order to establish several variables before commencement of the main study. As can be seen in Table 4-1 the first phase, done in order to confirm the FSL as a valid animal model of depression resulted the FSL rats being significantly more immobile than FRL rats with no differences in locomotor activity. We further found a relationship between maximal exercise intensity of FSL rats and age during pre-pubertal development (see 3.3). Using an equation obtained from the correlation between maximal exercise intensity and age we calculated low and moderate intensity and subjected animals to daily exercise during pre-pubertal development in order to establish the exercise intensity to be used in the main study. Behavioural analyses on exercised animals indicated no significant differences in immobility in the FST (see Table 4-1 Phase 2b), although low intensity significantly increased swimming behaviour in accordance to several studies reporting low intensity exercise to exert anti-depressive effects in the FST. No significant differences in the number of line crossings were observed in the open field test. Furthermore we compared different dosages of fluoxetine and established that only fluoxetine 5 mg/kg/day significantly decreased immobility in the FST early after treatment. Although fluoxetine 10 mg/kg/day did not alter behaviour in the FST, significant differences in locomotor activity were observed when compared to the vehicle control. We further demonstrated that the combination of fluoxetine 5mg/kg/day with low intensity exercise exerts anti-depressive-like effects (see Table 4-1 Phase 4) as indicated by a significant decrease in immobility in the FST when compared to the vehicle plus sedentary control. No changes were observed in locomotor activity.

Finally, we assessed whether low intensity exercise, fluoxetine 5 mg/kg/day and the combination of fluoxetine 5 mg/kg/day and low intensity exercise exerts long-lasting effects on behaviour and neurochemistry after a 26 day period consisting of normal housing and no treatment. We established that low intensity exercise and fluoxetine 5 mg/kg/day significantly improved depressive-like behaviour, significantly decreasing immobility and increasing climbing behaviour in the FST. We observed no differences in swimming behaviour in the FST or number of line crossings in the OFT. Further, data obtained from the augmentative strategy indicated no significant differences in any of

the behaviours assessed in the FST, although a significant decrease in the number of line crossing were observed in the OFT. Neurochemical analyses indicated a significant increase in SOD inhibition in all treatment groups compared to the vehicle +sedentary control. Data also indicated a significant decrease in hippocampal BDNF in the fluoxetine plus exercise group when compared to the vehicle plus exercise group. However no significant differences in hippocampal BDNF were observed in any of the other groups as well as no significant differences in lipid peroxidation and plasma corticosterone in any of the treatment groups.

Table 4-1: Summary of behavioural analyses as obtained in Phase 1, 2a, 2b, 3, 4 and the main study as well as neurochemical analyses. Phase 1, 2a, 2b, 3 and 4 had no neurochemical analyses done as these phases only served as pilot studies. No behavioural analyses was done in Phase 2a i.e. exhaustion test. PostND = postnatal day; no change = ↔; decrease = ↓; increase = ↑, significant difference between indicated groups = *. Exercise: Sed = Sedentary (no exercise), Low = Low intensity and Mod = Moderate intensity; Veh = Vehicle (Saline), 5 mg = fluoxetine 5 mg/kg/day and 10 mg = fluoxetine 10 mg/kg/day; 5 mg + low = Augmentation of fluoxetine 5 mg/kg/day with low intensity exercise; LMA = locomotor activity; BDNF = brain-derived neurotrophic factor; Cort = corticosterone; MDA = Malondealdehyde; SOD = superoxide dismutase. .

Phase of study			**Phase 1**	**Phase 2a**	**Phase 2b**		**Phase 3**		**Phase 4**	**Main study**		
Behavioural test age / Treatment period			**PostND60**	**PostND21 to PostND34**	**PostND35**		**PostND35**		**PostND35**	**PostND60**		
Comparison			**FSL *vs* *FRL***	**FSL**	**Low *vs* *Sed***	**Mod *vs* *Sed***	**5 mg *vs* *Veh***	**10 mg *vs* *Veh***	**5 mg + Low *vs* *Veh***	**Low *vs* *Veh***	**5 mg *vs* *Veh***	**5 mg + Low *vs* *Veh***
Behaviour	**FST**	**Immobility**	↑	Linear relationship of age and treadmill speed for VO_2max: y = 1.856x – 30.68	↔	↔	↓	↔	↓	↓	↓	↔
		Climbing	↓		↔	↔	↑	↔	↔	↑	↑	↔
		Swimming	↔		↑	↔	↔	↔	↑	↔	↔	↔
	OFT	**LMA**	↔		↔	↔	↔	↓	↔	↔	↔	↔
		Anxiety	↔		↔	↔	↔	↔	↔	↔	↔	↔
Neurochemistry	**Hippocampus**	**BDNF**								*↔	↔	*↔
		MDA								↔	↔	↔
		SOD								↑	↑	↑
	Plasma	**Cort**								↔	↔	↔

4.2 Final discussion and Conclusion

This study was successful in addressing the objectives described in §1.3:

Primary objective

We successfully demonstrated that the depressive-like behaviour of pre-pubertal male rats with a genetic predisposition to develop depression are vulnerable to exposure to both subchronic fluoxetine and low intensity exercise, and that these effects are long-lasting into early adulthood (see discussion below). In contrast to our working hypothesis the combination of fluoxetine and low intensity exercise did not yield lasting augmentation of antidepressant effects, but rather was ineffective by early adulthood. Locomotor activity, however, was affected into adulthood, as discussed below.

Secondary objectives

We demonstrated that the FSL rat is a valid animal model of depression under our experimental conditions. The data confirmed that the FSL rat displays depressive-like behaviour relative to FRL rats on PostND60 (Table 4-1), so that we used only FSL rats as a genetic animal model of depression in the remainder of the study. These data were in agreement with previous work (Overstreet et al. 2005, Liebenberg et al. 2010, Mokoena et al. 2015) and as expected.

We also demonstrated exercise intensity should increase with age, in particular during pre-pubertal development of the rodent. The study demonstrated that maximal exercise intensity (VO_2max) increases with age from PostND21 to PostND34 (§3.3). The correlation between speed (m/min) and age as well as the significant difference between maximal intensity on PostND21 and PostND34 suggest a linearly related increase in exercise intensity (VO_2max) across pre-pubertal development. This highlights the need of adapting exercise intensity (low or moderate) according to age, rather than keeping it constant throughout treatment as done in previously reported studies (de Almeida et al. 2013, Kim et al. 2003, Park, Lee & Oh 2013). It is the first time to our knowledge that a specific exercise regime for the treatment of depressive-like behaviour was developed for pre-pubertal FSL rats with the intensity adapted specifically to age. We were able to determine an equation to describe the increase in VO_2max over time for the pre-pubertal phase of the FSL rat. Since the FSL rat was inbred from Sprague Dawley rats, we suspect that this can be applied to Sprague Dawley rats in general, and most likely also relate to rat lines such as Wistar rats. It may be important for researchers to confirm such a correlation in their animals, particularly when different rodents are utilised, and also when different age groups are employed.

Lastly we demonstrated that fluoxetine as well as the combination of fluoxetine and exercise are effective in the treatment of pre-pubertal depression. Our data demonstrated that 14 days of exercise

in pre-pubertal rats does not affect immobility in the FST on PostND35 (i.e. early after treatment), and hence overall depressive-like behaviour (§3.3 & Table 4-1). However, we did observe a significant difference in swimming behaviour in the low intensity exercise group, indicating differential effects of exercise intensity on escape-related behaviour in the forced swim test. Enhanced swimming has also been associated with enhanced serotonergic neurotransmission (Cryan, Valentino & Lucki 2005), so that these results would suggest that pre-pubertal exercise may support serotonergic neurotransmission (Bjørnebekk, Mathé & Brené 2010, Blumenthal et al. 2007). According to previous research low intensity exercise is more beneficial to alleviate depressive-like behaviour in juvenile animals (de Almeida et al. 2013, Kim et al. 2003, Lou et al. 2008, Park, Lee & Oh 2013). We observed no differences in locomotor activity of exercised versus sedentary control animals (§3.3 & Table 4-1), confirming that the increase in swimming behaviour is not resulting from altered locomotor activity, but rather from altered psychomotor activity.

We found fluoxetine administration to pre-pubertal rats are only effective to decrease immobility in the FST at PostND35 when administered at a low dose of 5 mg/kg/day, as opposed to no effect seen when administered at a higher dose of 10 mg/kg/day. The latter higher dose of fluoxetine also significantly decreased locomotor activity in the OFT, which could blunt any psychomotor-related decrease in immobility. Since dopamine may be modulated by SSRIs (Smith et al. 2009), it is conceivable that this blunting effect could potentially be attributed to a decrease in dopamine neurotransmission as discussed later on. These findings, in addition to previous reports (Klomp et al. 2014), supported the use of the lower dose of fluoxetine (5 mg/kg/day) in the main study.

Lastly, the significant difference in immobility between the sedentary plus vehicle-treated control animals and low intensity exercise plus low dose fluoxetine-treated animals suggested that the augmentative strategy may be a viable option to explore in the main study. No significant differences were observed in the locomotor activity of these animals, confirming that the observed effect in the FST is psychomotor-related and not locomotor-related.

Results from the main study suggest that pre-pubertal monotherapy with low intensity exercise and fluoxetine (5 mg/kg/day) exert lasting anti-depressive effects. Although studies of long-lasting effects of exercise on depressive-like behaviour are limited, studies of lasting effects of adolescent fluoxetine treatment found results similar to that of the current study (Iniguez et al. 2014, Karpova et al. 2009). However studies conducted during early-life prior to puberty found significantly different results to that of the current study and of adolescent treatment (Yu et al. 2014, Ansorge et al. 2004, Ansorge, Morelli & Gingrich 2008). Early-life inhibition of the serotonin transporter have been found to increase immobility in the FST, reduce exploratory behaviour, increase anxiety, reduce aggression, and increase REM sleep and anhedonia, although positive outcomes such as decreased impulsivity and improved learning and memory have also been observed (Olivier et al. 2011, Lee, Lee 2012). These findings underlines the

SSRI paradox as mentioned by Olivier and colleagues (2011) which states that early-life and adult fluoxetine exposure leads to distinctive, mostly opposing outcomes (Olivier et al. 2011). These different outcomes could be attributed to the maturation of the serotonergic pathway as the serotonergic pathway is fully matured at the start of the pre-pubertal period (Murrin, Sanders & Bylund 2007), whereas the noradrenergic and dopaminergic pathways are still developing until early adolescence and therefore more vulnerable to insults (Murrin, Sanders & Bylund 2007). This could further explain differences in outcomes between early-life, pre-pubertal, adolescent and adult fluoxetine exposure. This supports the idea that the age (or neurodevelopmental stage) of insult during development results in differential behavioural outcomes (Andersen 2003).

However, the combination of fluoxetine with exercise during pre-pubertal development in stress-sensitive rats was ineffective in exerting lasting effects on depressive-like behaviour (i.e. the combination was not augmentative). This was not expected, since this combination has been proposed as an effective treatment option during pre-adolescence. The lack of anti-depressive-like behaviour in the FST could potentially be attributed to the significant decrease in locomotor activity later in life, which further suggests that there might be alterations on neurochemical level. The latter was, however, not explored in sufficient detail in the current study. Several studies have found alterations in locomotor activity after early-life increases in serotonin concentration (Olivier et al. 2011). That said, these alterations were linked to very early-life (PostND0-21) treatment with fluoxetine, suggesting that the augmentation of fluoxetine with exercise could cause alterations in early-life that are less beneficial to development than either fluoxetine or exercise alone and consequently resulting in behavioural deficits in early adulthood.

Our data also suggest that the addition of fluoxetine reduces BDNF levels as compared to exercise alone. The latter would also support findings of the current study in the FST, where the exercise alone, but not the combination of exercise plus fluoxetine, exerted antidepressant-like effects in the FST. We observed no significant differences in lipid peroxidation (MDA) potentially suggesting that oxidative damage does not explain behavioural changes observed in any of the treatment groups, although further studies are needed to establish this observation. We furthermore observed a significant increase in anti-oxidant capacity in the hippocampus of all treatment groups, suggesting a beneficial effect of all treatment groups. In fact, SOD is believed to play an important role in preventing oxidative damage; a finding in line with observations in depressed individuals (Bilici et al. 2001). No differences were observed in plasma corticosterone, suggesting that pre-pubertal exercise and fluoxetine do not cause lasting effects on the hypothalamic-pituitary-adrenal (HPA) axis. Taken together, although both fluoxetine and exercise have previously been shown to affect the biomarkers we examined, the post-intervention period of normal housing and no treatment (washout) could also result in some biomarkers returning to baseline levels. Furthermore stress during the forced swim procedure prior to decapitation and brain tissue dissection could also have influenced neurobiological results obtained as the same animals were used for both

behavioural and neurochemical testing 12 hours apart. Although this might be the case it is generally conceived that levels will return to baseline within hours after subjection to specifically the FST (Cryan, Valentino & Lucki 2005).

In the pilot studies, the FSL rats displayed a significantly lower immobility in the FST, as expected. That climbing, but not swimming behaviour of FSL rats were significantly less that that of the FRL rat, suggest that the depressive-like behaviour of the FSL rats are predominant due to decreased catecholaminergic neurotransmission and not to altered serotonergic neurotransmission. Interestingly, both fluoxetine and exercise alone displayed significantly higher climbing scores, and not swimming in the FST, also suggesting a predominantly catecholaminergic response with changes in serotonergic neurotransmission. Whether low dose monotherapy with fluoxetine during pre-adolescence alters the neurochemistry of the FSL rat to such an extent that its behaviour resembles that of the control FRL rat in early adulthood, needs to be explored. The behavioural and neurochemical analyses and outcomes of the current study will assist in formulating working hypotheses for further investigation. In this regard, the FST hints towards changes in serotonin, noradrenalin and dopamine neurotransmission, as reflected in swimming and climbing (Cryan, Markou & Lucki 2002, Cryan, Valentino & Lucki 2005), whereas locomotor activity in the OFT hints to the involvement of dopamine (Beninger 1983).

Pre-pubertal treatment with fluoxetine, exercise and the combination of fluoxetine and exercise leads to increased serotonergic neurotransmission in early-life. This could potentially mean that elevating serotonergic concentrations above a physiological range could lead to alterations in neurodevelopment, resulting in behaviour in adulthood different than that in early-life, as we have observed in the combined treatment group. This phenomenon may be explained by the equal but opposite hypothesis, postulating that the increased serotonin levels in early-life could down-regulate receptors, alter neurodevelopment and yield effects lasting into adulthood (Andersen 2003, Norrholm, Ouimet 2000). This is also supported by the clomipramine model of depression, according to which increased levels of serotonin in early-life result in depressive-like behaviour in adulthood (Vázquez-Palacios, Bonilla-Jaime & Velázquez-Moctezuma 2005). Although augmentation could lead to significantly improved neurochemical changes and behaviour in adults, it is not necessarily the case for juvenile treatment. In the latter case the responses suggest a dose-dependent effect. In support of this idea, it has been found that stress within normal physiological range promotes synaptic plasticity, whereas pathological (very high) levels lead to impaired structure and function (Andersen 2003).

Previous studies reporting on early-life interventions have found alterations in monoamine concentrations in adulthood, providing further support for the results obtained in the current study. Fluoxetine exposure during adolescence enhances reward processes linked to dopamine (Iniguez et al. 2014). A study conducted in our laboratory found pre-pubertal fluoxetine to increase basal levels of dopamine in later-life when compared to vehicle treated rats (Badenhorst 2014). This is a relevant finding, as dopamine has

been found to increase climbing behaviour in the FST (Page et al. 1999), potentially explaining the increased climbing behaviour observed in both the fluoxetine and low intensity exercise groups on PostND60. That said, decreased dopamine neurotransmission result in reduced locomotor activity (Beninger 1983), so that increased dopamine is expected to result in increased locomotor activity in the OFT. The novelty of the environment in the open field arena (i.e. upon first exposure of the animal to the arena) could also in part explain the lack of significant differences in locomotor activity observed in the current study (Olivier et al. 2011). Previous reports also found that peri-adolescent treatment with fluoxetine, decreases aggressive behaviour in adulthood, due to decreased dopamine neurotransmission (Yu et al. 2014). Furthermore, BDNF has been found to increase dopamine turnover (Martin-Iverson, Todd & Altar 1994) and therefore the decrease in hippocampal BDNF levels obtained in the augmented group could have resulted in deficits in dopamine concentration, ultimately leading to decreased locomotor activity in the OFT (Beninger 1983). Lastly, the importance of dopamine during development is also highlighted by the finding that altered dopaminergic signalling during pre-adolescence affects behaviour in adulthood, as seen in a study conducted in pre-adolescent rodents sub-chronically exposed to methylphenidate, resulting in the decreased responsiveness to cocaine's locomotor-activating effects in adulthood (Yu et al. 2014).

In conclusion, although studies conducted on early-life antidepressant treatment have often reported and focussed on adverse effects of early-life treatment, we now propose that the pre-pubertal period as a sensitive period in neurodevelopment that could lead to beneficial or detrimental neurochemical and behavioural effects in early adulthood. Furthermore the effects observed resulting from pre-pubertal treatment are dependent on the antidepressant dose and exercise intensity, as observed in a validated genetic animal model of depression. Accordingly, monotherapy with low dose fluoxetine or low intensity exercise during the pre-pubertal period in rodents genetically susceptible to stress, results in beneficial behavioural outcomes in early adulthood. Although the current study hints toward a role of monoaminergic neurotranmitter levels, the exact mechanism behind these findings, however, remains to be elucidated.

4.3 Recommendations

Despite the success in several aspects of the study, it is important to keep in mind the limitations in the present study. Several question raised by the current study warrants further investigation. Accordingly, the following recommendations have been made:

1. **Limitations of the study and recommendation for prospective studies:**
   - The inclusion of a control strain of rats in the main study, namely the FRL rat, may improve the interpretation of data, as well as aid in giving insight into differential effects of fluoxetine when compared to animals with a genetic predisposition to develop depression i.e. FSL rats.

Several studies focus on animals without depressive-like symptoms, so that combining this into one study could explain the role of genetic susceptibility with regards to lasting effects of early-life interventions.

- ❖ Only male FSL rats have been used in the current study due to the variability of results produced by the menstrual cycles of female rats. However the inclusion of female rats in studies could potentially increase the interpretation and translatability of the data to that of the human condition.
- ❖ In order to further improve the interpretation of the data it is also of importance to explore the age of onset of depressive-like behaviour in the FSL rat, specifically in our laboratory. Subjecting FRL and FSL rats to the FST and OFT on earlier occasions such as PostND21, 28 and 35 could further contextualise findings on PostND35.
- ❖ Separate groups of animals should be used for behavioural and neurochemical analyses in order to minimise variables as a result of stress from the FST as mentioned earlier. By removing the variability in data obtained after swim stress could improve interpretation of results.

2. **Studies to delineate the mechanisms underlying lasting effects, in particular the relationship between behaviour and specific neurochemical markers:**
   - ❖ Neurochemical analyses should be done on both PostND35 and PostND61 to assess neurochemical changes immediately after treatment as well as in later-life in order to assess differences of action on a neurochemical level, and thus aiding the interpretation of data.
   - ❖ Neurochemical analyses should be performed in order to unravel the neurobiological basis of the early and lasting effects of fluoxetine and exercise. This include the role of modulation of monoaminergic neurotransmission, inflammatory markers, oxidative stress and markers associated with neurogenesis and neuroplasticity (see §2.4).
   - ❖ Analyses should be expanded to include neuroimaging to observe neuroanatamical changes as well as immunohistochemistry to observe changes in neurogenesis and synaptogenesis following treatment.
   - ❖ Lastly, an extended battery of behavioural analyses, such as the sucrose preference test, morris water maze and the novel object recognition test, could assess anhedonia, memory and learning and recognition memory, respectively.
3. **Studies should be designed to establish why and when the effect of pre-pubertal treatment wears off after withdrawal. This is in order to explain why the combination therapy was ineffective in exerting lasting effects.**
   - ❖ Several of the above mentioned ideas could aid in explaining the reason behind this finding. However, behavioural and possibly neurochemical analyses on earlier (PostND45) as well as later stages (PostND90), in combination with data from PostND60 can further explain when

the lasting effects of pre-pubertal treatment wears off (fluoxetine plus exercise) or precipitates (exercise), such as has been observed in the current study. Furthermore whether the antidepressant-like effect is only present until early-adulthood or whether it lasts until late-adulthood.

ADDENDUM A

ADDITIONAL DATA

A.1 Material and Methods

A.1.1 Treadmill Familiarization

Animals were familiarized according to the method described in Chapter 3. The method was adapted from previous work (Gomes da Silva et al. 2012). Table A.1-1 depicts each postnatal day and the increments in speed (intensity) made throughout the 10 minutes of familiarisation. For example, on PostND16 pups were placed on the treadmill (0m/min) to allow pups to become habituated to the environment of the treadmill. After three minutes the treadmill was switched on (2m/min – lowest speed possible) and pups monitored. Pups were moved to the front of the treadmill when approaching the back of the treadmill to familiarise them to the idea of running. During the last 2 minutes pups were left to become familiar to the shocking grid.

Table A.1-1 Familiarization Protocol as adapted from (Gomes da Silva et al. 2012)

Time point (minutes)	PostND16	PostND17	PostND18	PostND19	PostND20
0-1	0 m/min	2 m/min	2 m/min	2 m/min	2.5 m/min
1-2	0 m/min	2 m/min	2 m/min	2 m/min	2.5 m/min
2-3	0 m/min	2 m/min	2 m/min	2 m/min	2.5 m/min
3-4	2 m/min	2 m/min	2.5 m/min	3 m/min	3.5 m/min
4-5	2 m/min	2 m/min	2.5 m/min	3 m/min	3.5 m/min
5-6	2 m/min	2.1 m/min	Rest	3 m/min	3.5 m/min
6-7	2 m/min	2.2 m/min	2.5 m/min	4 m/min	4.5 m/min
7-8	2 m/min	2.3 m/min	3 m/min	4 m/min	4.5 m/min
8-9	2 m/min	2.4 m/min	3 m/min	4 m/min	4.5 m/min
9-10	2 m/min	2.5 m/min	3 m/min	4.5 m/min	

A.1.2 Molecular Studies

Molecular studies were performed on plasma as well as hippocampal tissue according to methods described below.

The hippocampus is one of the most notably affected brain regions in MDD (Duman, Malberg & Thome 1999, Femenía et al. 2012) mainly due to high vulnerability particularly in early-life (Duman, Malberg & Thome 1999) to oxidative stress (Patki et al. 2013), prolonged increases in plasma corticosterone (Pluchino et al. 2013) and resulting alterations in neurotrophins such as BDNF (Dwivedi 2009).

All of the abovementioned stress-induced alterations have been found to be reversed by fluoxetine treatment and exercise in patients with MDD during adulthood.

A.1.2.1 Hippocampal tissue Preparation

On PostND61 (i.e. 27 days after the last treatment and/or exercise session) rats were euthanised by means of decapition, the whole brain removed and placed in ice-cold double distilled water (ddH_2O), where after the hippocampi were dissected out on an ice-cooled dissection slab (Chiu et al. 2007). The hippocampi were then snap frozen in liquid nitrogen and stored at -80^{o}C until used.

A.1.2.2 Blood Collection

Immediately after decapitation trunk blood was collected in pre-chilled, 4 ml vacutainer tubes (SGVac, containing lithium heparin solution as anticoagulant), centrifuged at 3000 rpm at 4°C for 10 min, and the plasma stored at -80°C until the day of analysis (Viljoen, Brand & Smit 2012).

A.1.2.3 Corticosterone

Blood samples were collected as described above (see *Blood Collection)*. Stored plasma samples were thawed on ice on the day of analysis and prepared according to a method previously described (Viljoen, Brand & Smit 2012). Briefly, sample preparation was done using screw-capped glass tubes (diameter 10 mm, height 100 mm, volume ± 8 ml) adding 500 µl of the sample (plasma), 50 µl of the internal standard (dexamethasone 1 µg/ml) and 5 ml dichloromethane. Standards (water) were made using identical screw-capped glass tubes adding 500 µl of water (containing 500ng/ml corticosterone stock solution), 50 µl of the internal standard (dexamethasone 1 µg/ ml) and 5 ml of dichloromethane. Plasma samples and water standards were vortexed for 2 minutes and centrifuged at 3,000 rpm for 15 minutes. Thereafter the upper layer was removed and the lower organic layer transferred to conical tubes. Nitrogen was used at room temperature to evaporate the liquid from the preparation. A volume of 150 µl of mobile phase was used to reconstitute each sample, which was then transferred into glass inserts and placed in vials. Finally the vials were placed in the auto-sampler and analysis done by ultra-violet (UV)-high pressure liquid chromatography (HPLC) as described below.

A.1.2.4 HPLC-method

Samples were prepared as described above. The method used was previously described by Viljoen and colleagues (2012) (Viljoen, Brand & Smit 2012). The constituents of the mobile phase were: distilled water; acetonitrile and glacial acetic acid (65:35:0.05, v/v). The pH of the mobile phase ranged from 4.10 to 4.20. A flow rate of 1.0 ml/min was used. The sample injection volume was 100 µl. The eluent was monitored at a wavelength of 245 nm by the diode array detector. A run time of approximately 15 minutes was evident for each sample in a temperature controlled room (24°C) [63].

A.1.2.5 **Lipid Peroxidation**

Hippocampus tissue samples were collected and prepared as described above (see *Hippocampal tissue Preparation*). Lipid peroxidation was determined using a lipid peroxidation, malondealdehyde (MDA), colorometric assay kit from Biovision™, and the assay was performed according to the manufacturer's protocol. Briefly, 10 mg wet weight of hippocampal tissue was used to prepare a homogenate of 10mg sample homogenised on ice in 300µl of MDA lysis buffer according to the manufacturer's instructions. Briefly, 200 µl of the supernatant (centrifuged homogenate) was pipetted into a centrifugable tube. 600 µl of thiobarbituric acid (TBA) was added and incubated in glass vials at 95°C for 60 minutes. The vials were cooled on ice to room temperature. 200 µl of the reaction mixture was pipetted into a 96-well microplate for spectrophotometric analysis at 532 nm. Data are expressed as nmol malondialdehyde formed/mg protein calculated with the following equation:

C = [(A/mg)] x 4 x D = nmol/mg

where: C = concentration, A = sample MDA amount from the standard curve (in nmol), mg = original amount of tissue used, 4 = Correction for using 200 µl of the 800 µl reaction mix, D = Dilution factor

A.1.2.6 **Superoxide dismutase**

Hippocampal tissue samples were collected and prepared as described above (see *Hippocampal tissue Preparation)*. Percentage SOD inhibition was determined using a SOD Activity Assay Kit from Biovision™ and the assay was performed according to the manufacturer's protocol. Briefly, 10 mg wet weight of hippocampal tissue was used to prepare a homogenate containing the hippocampal tissue (sample) homogenised in ice cold 0.1M Tris/HCl pH 7.4, according to the manufacturers' instructions. Briefly, 20 µl of supernatant (centrifuged homogenate) was pipetted with 200 µl of water soluble tetrazolium (WST) working solution and 20 µl of enzyme working solution were pippeted into a 96-well plate. Spectrophotometric analyses at 450 nm in a microplate reader were done after incubating the plate at 37°C for 20 minutes. Data are expressed as SOD activity (inhibition rate %), calculated with the following equation:

SOD activity (inhibition rate %) was then calculated using the following equation:

$$\text{SOD activity \%} = \frac{(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})}{(A_{blank1} - A_{blank3})} \times 100$$

where the samples and blank are described in the table below:

Table A.1-2: Description of equation. WST = water soluble tetrazolium.

	Sample	Blank 1	Blank 2	Blank 3
Sample Solution	20 µl	-	20 µl	-
ddH_20	-	20 µl	-	20 µl
WST Working solution	200 µl	200 µl	200 µl	200 µl
Enzyme Working solution	20 µl	20 µl	-	-
Dilution Buffer	-	-	20 l	21 l

A.1.2.7 **BDNF**

Hippocampal tissue samples were collected and prepared as described above (see *Hippocampal issue Preparation)*. Hippocampal BDNF levels were determined using an enzyme-linked immunosorbent assay (ELISA) BDNF kit (Thermo Scientific) and the assay was performed according to the manufacturers' protocol. Briefly, 10 mg of hippocampal tissue was used to prepare a homogenate as previously described by Kolbeck and colleagues (1999) (Kolbeck et al. 1999). 100 µl of the supernatant (centrifuged homogenate) was transferred to a 96-well plate coated with anti-BDNF (1:1000). Plates were incubated for 2.5 hours at room temperature, after which the solution was discarded and the plate washed 4 times with 1x wash buffer. 100 µl of 1X prepared biotinylated antibody were added to each well and incubated for 1 hour at room temperature with gentle shaking. The wash step was repeated after incubation. 100 µl of Streptavidin-HRP solution was then pipetted to each well and incubated for 45 minutes with gentle shaking and the wash step repeated. Finally, 100 µl of tetramethyl benzidine (TMB) substrate was pipetted to each well and incubated for 30 minutes at room temperature in a dark room with gentle shaking. Spectrophotometric analysis at 450 nm in a plate reader after a colour reaction with TMB was then quantified and the results obtained from a standard curve plotted according to the manufacturers' instructions.

A.2 Results and discussion

A.2.1 Phase 1, Validation of the FSL as an animal model of depression (see Chapter 1 for study layout)

Figure A.2-1 represents behavioural data of Phase 1 (see Chapter 1) on PostND60 in FRL and FSL rats

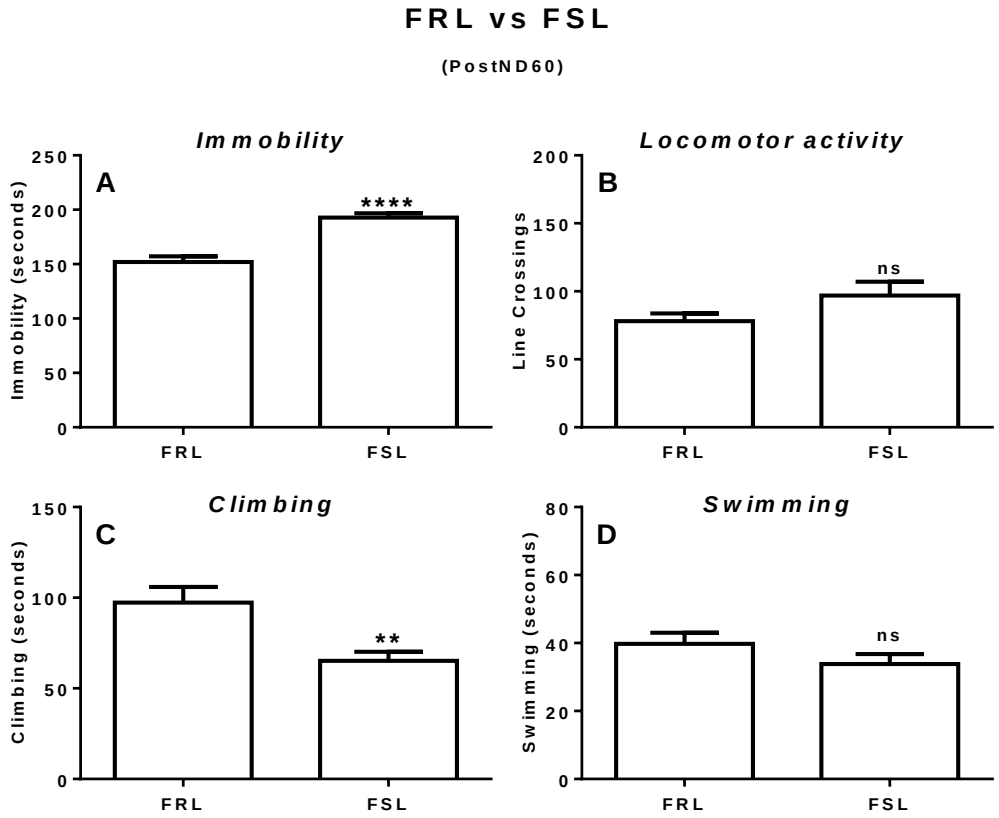

Figure A.2-1: Pilot study on the behaviour of FRL and FSL rats on PostND60 in the FST.

(A) Immobility in the FST on PostND60 following normal housing and no treatment of FRL rats compared to FSL rats. (B) Number of line crossings in the OFT. (C) Climbing in the FST. (D) Swimming in the FST. Data points represent the mean ±SEM, n = 12. Statistical analyses are reported in the text, with ns = non-significant, ** $p < 0.01$ **** $p < 0.0001$.

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-1 ($p > 0.05$ in all cases), so that the Student's t test could be applied as appropriate.

In Figure A.2-1A an unpaired Student's t-test of the data indicated that FSL rats display significantly enhanced immobility relative to FRL rats (151.9 ± 5.296 vs 192.8 ± 4.010 $p<0.0001$) on PostND60. No statistically significant differences were observed in locomotor activity (Figure A.2-1B) between FSL and FRL rats (78.17 ± 5.500 vs 96.83 ± 10.33, ns = non-significant). In Figure A.2-1C an unpaired students' t-test of the data indicated that climbing behaviour of FSL rats compared to FRL rats was significantly

decreased on PostND60 (97.36 ± 8.552 vs 65.14 ± 5.051, p=0.00947) although no differences were observed in swimming behaviour (39.77 ± 3.282 vs 33.84 ± 2.924, non-significant) see Figure A.2-1D.

The data in Figure A.2-1A demonstrate that FSL rats demonstrated significantly more depressive-like behaviour (immobility) in the FST when compared to FRL rats in agreement with previous reports (Overstreet et al. 2005, Mokoena et al. 2015, Liebenberg et al. 2010). The difference in immobility is attributed to the FSL rat engaging in significantly less escape related behaviour i.e. climbing behaviour (Figure A.2-1C) in the FST than the FRL, whereas no differences were observed in swimming behaviour (Figure A.2-1D). The reduced immobility in FSL rats as compared to FRL rats is not due to differences in locomotor activity (Figure A.2-1B), so that immobility data can be explained by differences in psychomotor ativity (Cryan, Valentino & Lucki 2005). Others reported differences in locomotor activity of FSL and FRL rats (Overstreet et al. 2005).

Figure A.2-2 represents behavioural data of Phase 1 (see Chapter 1) on PostND60 in FRL and FSL rats

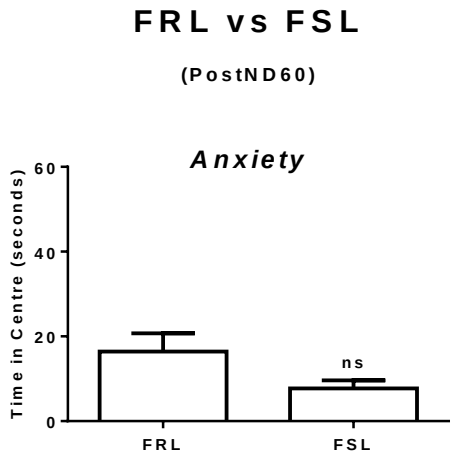

Figure A.2-2: Pilot study on the behaviour of FRL and FSL rats on PostND60 in the OFT
Time spent in centre square of the open field arena. Data points represent the mean ±SEM, n = 12. Statistical analyses are reported in the text, with ns = non-significant.

The Shapiro-Wilks test indicated that the assumption of normality was true for the data set in Figure A.2-2 ($p > 0.05$ in all cases), so that the Student's t test could be applied as appropriate.

Figure A.2-2 an unpaired Student's t-test of the data indicated no significant difference in time spent in the centre square of the open field arena between FRL and FSL rats (16.41 ± 4.326 vs 7.713 ± 1.924, non-significant).

Anxiety-like behaviour as measured in the open field arena was not significantly different, a finding in agreement with previous reports suggesting that the FSL rat is an animal model of depression without anxiety-like traits (Malkesman et al. 2006).

A.2.2 **Phase 2a: Exhaustion test in order to indirectly determine VO_2max in pre-pubertal FSL rats (see Chapter 1 for study layout)**

Data obtained as presented in 3.3 were used to determine an equation from which the maximal treadmill speed (m/min) for each day could be determined. The maximal speed (100 %) in turn was used to determine the moderate (70 %) as well as low intensities (55 %) for each postnatal day.

Table A.2-1: Maximal exercise intensities as determined from the equation: y = 1.855x – 30.58, for each day during pre-adolescent development as well as the moderate and low intensities to be used in the exercise regimen.

	Intensity (m/min)		
PostND (age)	100% (maximal)	70% (moderate)	55% (low)
21	8.4	5.9	4.6
22	10.2	7.2	5.6
23	12.1	8.5	6.6
24	13.9	9.8	7.7
25	15.8	11.1	8.7
26	17.7	12.4	9.7
27	19.5	13.7	10.7
28	21.4	15.0	11.7
29	23.2	16.3	12.8
30	25.1	17.5	13.8
31	26.9	18.8	14.8
32	28.8	20.1	15.8
33	30.6	21.4	16.8
34	32.5	22.7	17.9

A.2.3 **Phase 2b: Effects of different intensities of treadmill exercise during pre-pubertal development on depressive-like behaviour in FSL rats (see Chapter 1 for study layout)**

Figure A.2-3 represents escape related behaviours as measured in the FST as well as anxiety-like behaviour as measured in the OFT in FSL rats on PostND35.

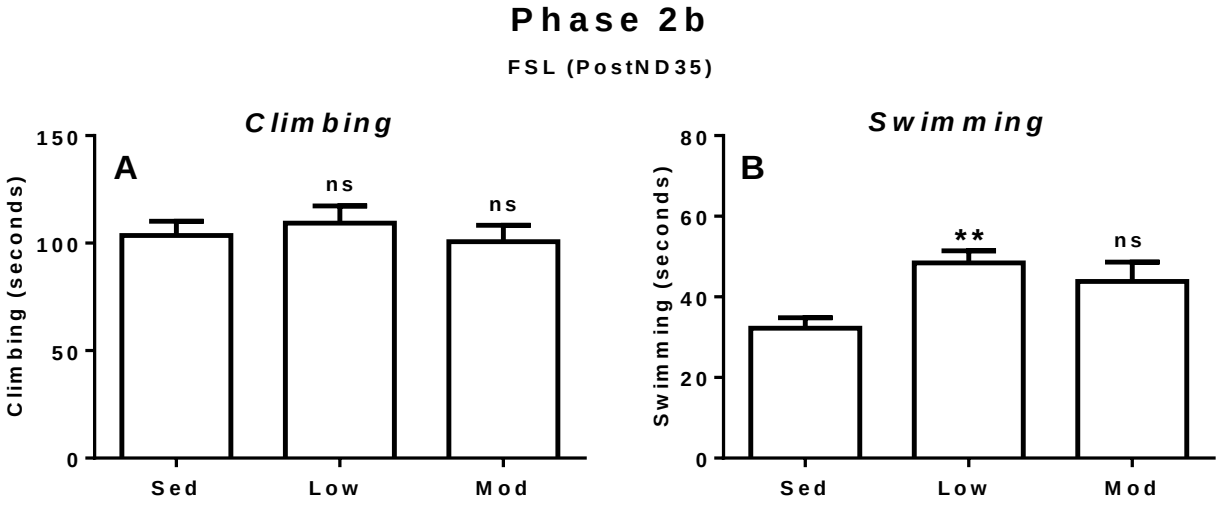

Figure A.2-3: Pilot study on behaviour in the FST in exercise treated FSL rats on PostND35.

(A) Climbing behaviour of FSL rats in the FST on PostND35 following no (sedentary) (n = 16), low (n = 14) and moderate (n = 11) intensity exercise. (B) Swimming behaviour of the abovementioned treatment groups on PostND35. Data points represent mean ±SEM. Statistical analyses are reported in text, with ns = non-significant, **p < 0.01.

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-3 ($p > 0.05$ in all cases), so that the ordinary one-way ANOVA could be applied as appropriate.

In Figure A.2-3A an ordinary One-way ANOVA of the data ($F[2, 39] = 0.3149$, $p = 0.7317$) indicated no significant interaction regarding climbing between exercise groups. In Figure A.2-3B an ordinary One-way ANOVA of the data ($F [2,39] = 6.957$, $p = 0.0026$) followed by Tukey's post hoc analysis indicated a significant increase in swimming behaviour of the low intensity exercise group when compared to the sedentary control ($p < 0.01$). No significant differences were observed in swimming behaviour of the moderate intensity group compared to the sedentary control (non-significant).

The data in Figure A.2-3A demonstrate that neither low nor moderate intensity exercise for 14 days affected climbing behaviour in the FST on PostND35. Swimming behaviour (Figure A.2-3B) however was significantly increased in the low intensity group compared to the sedentary control indicating an increase in serotonergic neurotransmission (Cryan, Valentino & Lucki 2005), which is in agreement with previous reports (Blumenthal et al. 2007, Bjørnebekk, Mathé & Brené 2010). Moderate intensity did not

significantly affect swimming behaviour suggesting differential effects of exercise intensity during pre-pubertal development which is in agreement with previous reports (de Almeida et al. 2013).

Figure A.2-4 represents anxiety-like behaviour as measured in the OFT in FSL rats on PostND35.

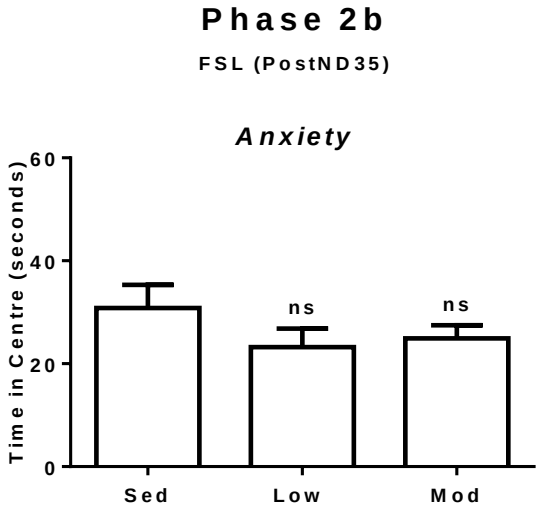

Figure A.2-4: Pilot study on behaviour in the OFT in exercise treated FSL rats on PostND35

Time spent in centre square of the open field arena on PostND35 following no (sedentary) (n = 16), low (n = 14) and moderate (n = 11) intensity exercise. Data points represent mean ±SEM. Statistical analyses are reported in text, with ns = non-significant,

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-4 ($p > 0.05$ in all cases), so that the ordinary one-way ANOVA could be applied as appropriate.

In Figure A.2-4 an ordinary One-way ANOVA of the data ($F[2, 38] = 1.132$, $p = 0.3329$) indicated no significant interaction regarding time spent in centre of the open field arena between exercise groups (non-significant).

Anxiety-like behaviour was not significantly affected in any of the exercised groups on PostND35.

A.2.4 **Phase 3: Effects of different dosages of fluoxetine during pre-pubertal development on depressive-like behaviour in FSL rats (see Chapter 1 for study layout)**

Figure A.2-5 represents escape related behaviours as measured in the FST as well as anxiety-like behaviour as measured in the OFT in FSL rats on PostND35.

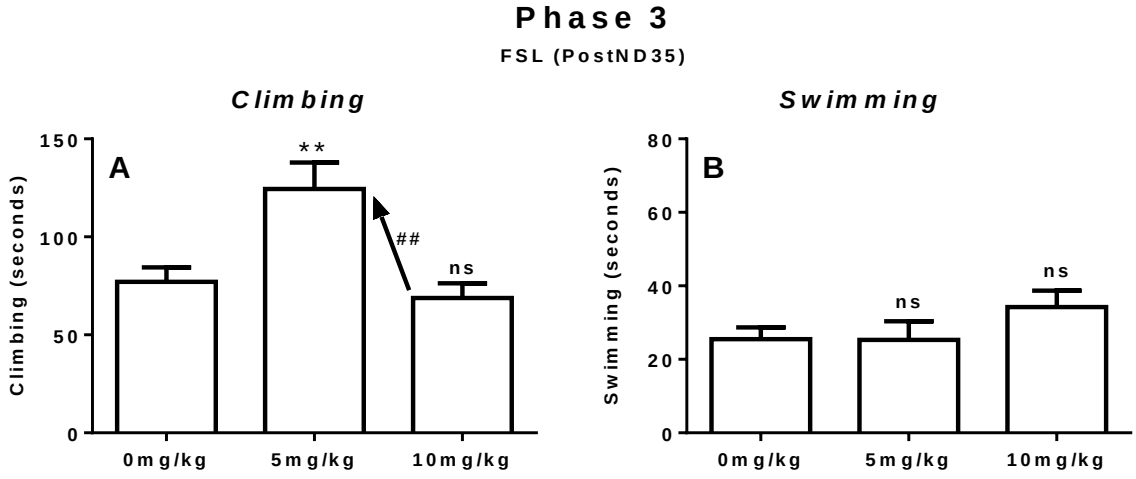

Figure A.2-5: Pilot study on behaviour in the FST in fluoxetine treated FSL rats on PostND35.

(A) Climbing behaviour of FSL rats in the FST on PostND35 following vehicle (n = 8), fluoxetine 5 mg/kg/day (n = 7) and fluoxetine 10 mg/kg/day (n = 7) intensity exercise. (B) Swimming behaviour of the abovementioned treatment groups on PostND35. Data points represent mean ±SEM. Statistical analyses are reported in text, with ns = non-significant, **p < 0.01 vs control, ## < 0.01 vs indicated test group.

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-5 ($p > 0.05$ in all cases), so that the ordinary one-way ANOVA could be applied as appropriate.

In Figure A.2-5A an ordinary One-way ANOVA of the data ($F[2, 19] = 9.297$, $p = 0.0015$) followed by Tukey's post hoc analysis indicated a significant difference regarding climbing between fluoxetine 5 mg/kg/day and the vehicle control ($p < 0.01$) as well as the fluoxetine 10 mg/kg/day ($p < 0.01$). No significant difference was observed between the fluoxetine 10 mg/kg/day and the vehicle control. In Figure A.2-5B an ordinary One-way ANOVA of the data ($F [2,19] = 1.431$, $p = 0.2637$) indicated no significant difference regarding swimming behaviour between treatment groups.

The data in Figure A.2-5A demonstrates that although fluoxetine 10 mg/kg/day did not significantly alter climbing behaviour in the FST on PostND35, fluoxetine 5 mg/kg/day did significantly increase climbing behaviour when compared to the saline control as well as the fluoxetine 10 mg/kg/day groups. Interestingly, although fluoxetine increases serotonin neurotransmission, swimming behaviour (Figure A.2-5B), which is associated with serotonergic neurotransmission, was unaffected by either dosages.

Similar results were previously observed in Wistar Kyoto rats with fluoxetine 5 mg/kg/day and 10 mg/kg/day, where only 20 mg/kg/day was effective in exerting swimming behaviour. Swimming was increased dose-dependently in Sprague dawley rats, suggesting differential effects depending on rat strain (López-Rubalcava, Lucki 2000). Furthermore, except for low animal numbers we do not have an explanation for the finding of the current study.

Figure A.2-6 represents anxiety-like behaviour as measured in the OFT in FSL rats on PostND35.

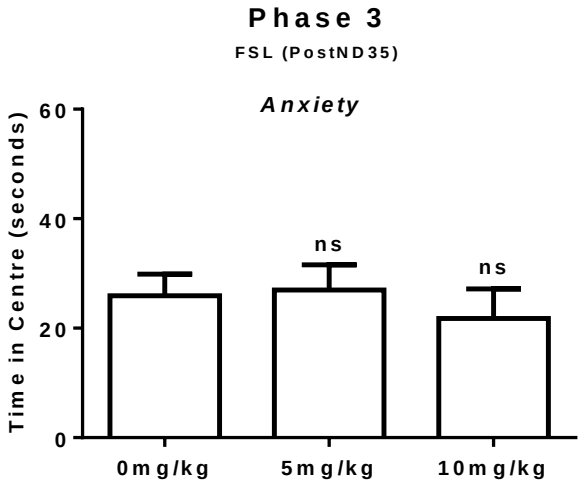

Figure A.2-6: Pilot study on behaviour in the OFT in fluoxetine treated FSL rats on PostND35.
Time spent in centre square of the open field arena on PostND35 following vehicle (n = 8), fluoxetine 5 mg/kg/day (n = 7) and fluoxetine 10 mg/kg/day (n = 7) intensity exercise. Data points represent mean ±SEM. Statistical analyses are reported in text, with ns = non-significant

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-6 ($p > 0.05$ in all cases), so that the ordinary one-way ANOVA could be applied as appropriate.

In Figure A.2-6 an ordinary One-way ANOVA of the data ($F[2,19] = 0.3428$, $p = 0.7141$) indicated no significant interaction regarding time spent in centre of the open field arena between treatment groups.

Anxiety-like behaviour as measured in the open field arena was not significantly affected in any of the exercised groups on PostND35.

A.2.5 **Phase 4: Effect of the augmentation of fluoxetine with low intensity exercise during pre-pubertal development on depressive-like behaviour in FSL rats (see Chapter 1 for study layout)**

Figure A.2-7 represents escape related behaviours as measured in the FST as well as anxiety-like behaviour as measured in the OFT in FSL rats on PostND35.

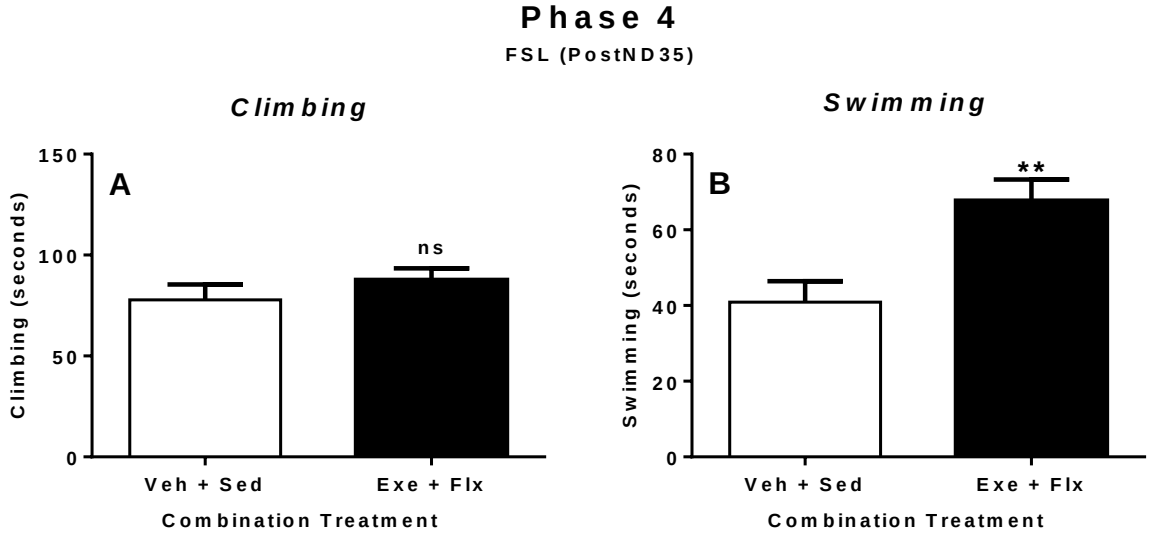

Figure A.2-7: Pilot study on behaviour in the FST and OFT in fluoxetine combined with exercise treated FSL rats on PostND35.

(A) Climbing behaviour of FSL rats in the FST on PostND35 following vehicle plus sedentary (n = 12), and low intensity exercise plus fluoxetine 5 mg/kg/day (n = 12) treatment. (B) Swimming behaviour of the abovementioned treatment groups on PostND35. (C) Time spent in centre square of the open field arena on PostND35. Data points represent mean ±SEM. Statistical analyses are reported in text, with ns = non-significant, **$p < 0.01$.

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-7 ($p > 0.05$ in all cases), so that the Student's t test could be applied as appropriate.

In Figure A.2-7A an unpaired Student's t-test of the data indicated no statistically significant difference regarding climbing behaviour in the FST between fluoxetine plus exercise and the vehicle control on PostND35 (77.83 ± 7.581 vs 87.71 ± 5.724, non-significant). In Figure A.2-7B an unpaired Students' t-test of the data indicated a statistically significant difference regarding swimming behaviour in the FST between fluoxetine plus exercise and the vehicle control (40.88 ± 5.562 vs 67.73 ± 5.569, $p < 0.01$) on PostND35.

The data in Figure A.2-7A demonstrate that the augmentation of fluoxetine 5 mg/kg/day with low intensity exercise did not significantly alter climbing behaviour in the FST on PostND35. The

augmentation did however significantly increase swimming behaviour when compared to the saline plus sedentary control group (Figure A.2-7B). The increase in escape related behaviour in the augmented group could therefore be attributed to an increase in serotonergic neurotransmission as serotonin is known to increase swimming behaviour (Cryan, Valentino & Lucki 2005).

Figure A.2-8 represents anxiety-like behaviour as measured in the OFT in FSL rats on PostND35.

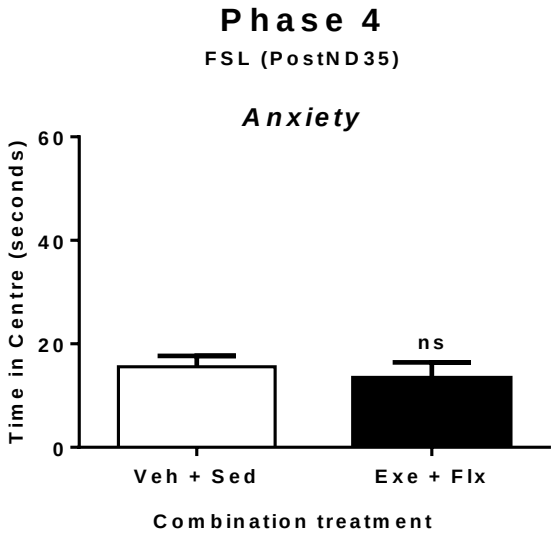

Figure A.2-8: Pilot study on behaviour in the OFT in fluoxetine combined with exercise treated FSL rats on PostND35.

Time spent in centre square of the open field arena on PostND35 following vehicle plus sedentary (n = 12), and low intensity exercise plus fluoxetine 5 mg/kg/day (n = 12) treatment. Data points represent mean ±SEM. Statistical analyses are reported in text, with ns = non-significant.

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-8 ($p > 0.05$ in all cases), so that the Student's t test could be applied as appropriate.

In Figure A.2-8 an unpaired Student's t-test of the data indicated no statistically significant difference regarding anxiety-like behaviour in the FST between fluoxetine plus exercise and the vehicle control on PostND35 (15.58 ± 2.092 vs 13.36 ± 3.088, non-significant).

Anxiety-like behaviour as measured in the open field arena was not significantly affected on PostND35.

A.2.6 Main Study: Effect of the fluoxetine, exercise and the augmentation of fluoxetine with low intensity exercise during pre-pubertal development on anxiety-like behaviour in FSL rats (see Chapter 1 for study layout)

Figure A.2-9 represents anxiety-like behaviour as measured in the OFT in FSL rats on PostND60.

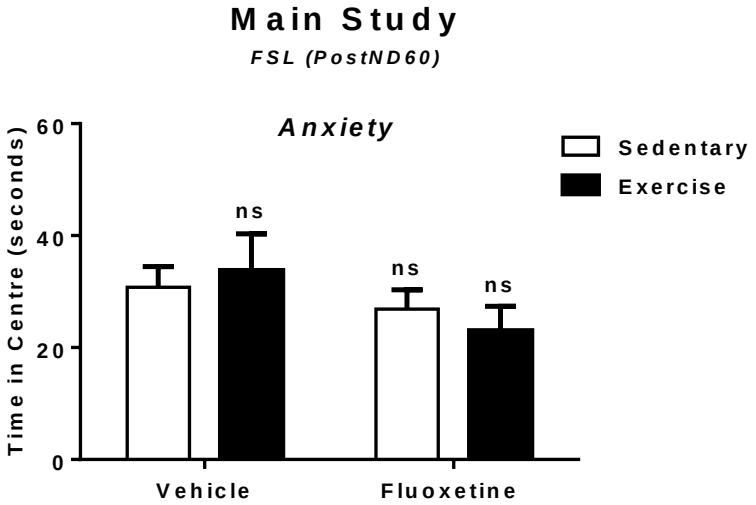

Figure A.2-9: Main Study time spent in centre square as measured in the open field test.

Time spent in centre square in the OFT on PostND60, after 26 days washout following pre-pubertal treatment with low intensity exercise (n = 12), fluoxetine 5 mg/kg/day (n = 12) and the augmentation of fluoxetine 5 mg/kg/day with low intensity exercise (n = 12) compared to the vehicle control (n=12). Data points represent the mean ±SEM. Statistical analyses are reported in the text, with ns = non significant.

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-9 ($p > 0.05$ in all cases), so that the ordinary two-way ANOVA could be applied as appropriate.

In Figure A.2-9 the two-way ANOVA of the data ($F [1,44] = 0.5363$; $p = 0.4678$) indicated no statistically significant interaction between drug treatment and lifestyle intervention regarding time spent in centre square on PostND60 indicating no difference in anxiety-like behaviour between any of the groups.

A.2.7 Main Study: Effect of the fluoxetine, exercise and the augmentation of fluoxetine with low intensity exercise during pre-pubertal development on hippocampal BDNF levels in FSL rats (see Chapter 1 for study layout)

Figure A.2-10 represents hippocampal BDNF in FSL rodents on PostND61.

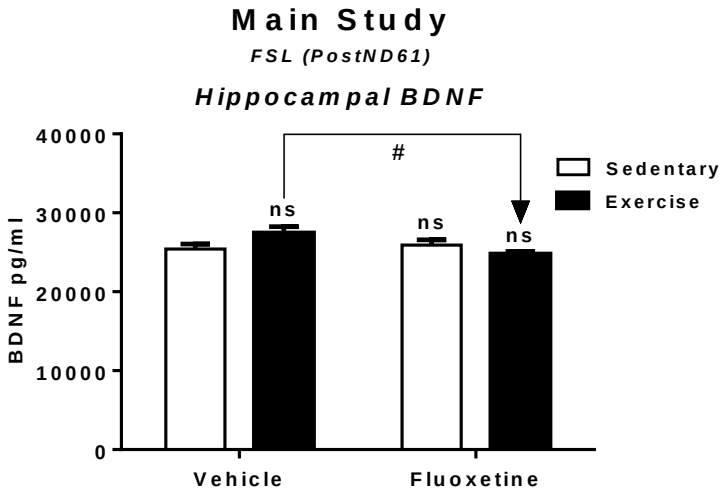

Figure A.2-10: Hippocampal BDNF levels (pg/ml) levels on PostND61 in pre-pubertal FSL rats treated with low intensity exercise, fluoxetine 5 mg/kg/day and the combination of fluoxetine and exercise.
Hippocampal levels of BDNF (pg/ml) on PostND61, after 27 days washout following treatment with low intensity exercise plus vehicle (n = 10) fluoxetine 5 mg/kg/day plus sedentary (n = 10) and the combination of exercise and fluoxetine (n = 10) when compared to a vehicle plus sedentary control (n = 10) as measured with a Rat BDNF ELISA kit (Thermo Scientific). Data points represent the mean ±SEM. Statistical analyses are reported in the text, with ns = non-significant vs control, # p = 0.05 vs indicated test group.

The Shapiro-Wilks test indicated that the assumption of normality was true for all data sets in Figure A.2-10 ($p > 0.05$ in all cases), so that the ordinary two-way ANOVA could be applied as appropriate.

In Figure A.2-10 a two-way ANOVA ($F [1, 35] = 6.030$; $p = 0.0192$) of the data indicated a statistically significant interaction between drug treatment and lifestyle intervention. The Tukey post hoc analyses multiple comparison indicated no significant differences in BDNF levels observed in all treatment groups compared to the sedentary control, however the addition of fluoxetine to exercise, significantly decreased BDNF levels as compared to the exercise alone ($p < 0.05$).

Data obtained from Figure A.2-10 demonstrate that hippocampal levels of BDNF on PostND61 in FSL rats are not altered by pre-pubertal exposure to any of the treatment strategies as compared to the saline plus sedentary control, following normal housing and no treatment for 27 days. These findings are in line with previous research that found the increase in BDNF following chronic exercise to only last for a maximum of 10 - 14 days (Vivar, Potter & van Praag 2013), before returning to baseline. Secondly the stress during the forced swim procedure prior to decapitation and brain tissue dissection could also have

decreased BDNF levels to baseline levels (Russo-Neustadt et al. 2004), although this would have been comparable for all groups. In this regard it has been reported that both exercise and fluoxetine can lead to increased BDNF transcription in the rat hippocampus of both juvenile and adult animals (Marais, Stein & Daniels 2009, de Almeida et al. 2013, Lou et al. 2008, Soya et al. 2007). However, to the best of our knowledge no study has yet subjected pre-pubertal FSL rats to treatment and tested them in early adulthood.

Our data also indicate that the addition of fluoxetine reduces BDNF levels as compared to exercise alone. The latter would also support findings of the current study in the FST, where the exercise alone, but not the combination of exercise plus fluoxetine, exerted antidepressant-like effects (as described in §3.3). These are interesting observations when taking into account that increased serotonin in early-life due to the predominant effect of exercise and fluoxetine on serotonergic neurotransmission has been found to alter the expression of neurotrophins and consequently neurogenesis and synaptogenesis (Kepser, Homberg 2015). Furthermore BDNF has been found to support dopamine turnover (Martin-Iverson, Todd & Altar 1994), so that decreased BDNF may be associated with deficits in dopamine neurotransmission. This is also in line with our findings of decreased locomotor activity in the combined treatment group (Figure 3-3B), particularly considering that reduced dopamine concentration is associated with decreased locomotor activity (Beninger 1983). Contrary to our data, it has also been reported that the combination of exercise and fluoxetine may lead to larger hippocampal BDNF mRNA levels than either intervention alone (Russo-Neustadt et al. 2004), but then these data do not reflect the current scenario of pre-pubertal treatment and lasting effects into adulthood.

Several researchers also reported increased neurogenesis and synaptogenesis following early-life low intensity exercise (de Almeida et al. 2013, Lou et al. 2008). Therefore it is is conceivable that increased monoamines, enhanced neurogenesis, increased cell survival, synaptic plasticity and enhanced vascular function following exercise (van Praag 2009) could potentially result in beneficial lasting effects.

A.2.8 Main Study: Biomarkers of depression on PostND61 in pre-pubertal FSL rats treated with low intensity exercise, fluoxetine 5 mg/kg/day and the combination of fluoxetine and exercise.

Table A.2-3 represents levels of biomarkers in plasma and hippocampi on PostND61 in FSL rats.

Table A.2-2: Biomarkers of depression on PostND61 in pre-pubertal FSL rats treated with low intensity exercise, fluoxetine 5 mg/kg/day and the combination of fluoxetine and exercise.

(Cort): Plasma levels of corticosterone pg/ml tissue on PostND61, after 27 days washout following treatment with low intensity exercise + vehicle (n = 12) fluoxetine 5 mg/kg/day + sedentary (n = 12) and the combination of exercise and fluoxetine (n = 12) when

compared to a vehicle + sedentary control (n = 12). (MDA): Hippocampal levels of lipid peroxidation (MDA) nmol/mg tissue on PostND61. (SOD): Hippocampal SOD activity (SOD % inhibition) on PostND61. Data points represent the mean ± SEM and n =10. Statistical analyses are reported in the text, with ns = non-significant ** $p < 0.01$ vs control, *** $p < 0.001$ vs control, **** $p = 0.0001$ vs control.

	Vehicle			**Fluoxetine**			
	Sedentary	**Exercise**		**Sedentary**		**Exercise**	
Cort	14079.2 ± 2738.8	18799.6 ± 3502.1	**Ns**	9460.2 ± 976.2	**ns**	15004.8 ± 3179.5	**ns**
MDA	12.6 ± 1.3	15.1 ± 0.8	**Ns**	13.3 ± 0.9	**ns**	12.6 ± 0.9	**ns**
SOD	73.8 ± 3.4	90.2 ± 1.1	********	90.2 ± 1.73	*******	87.3 ± 2.4	******

The assumption of normality of all data sets, except SOD, was met as analysed using the Shapiro-Wilks test as an analysis of variance for normality. Non-parametric analyses were therefore applied to the SOD data.

For corticosterone data in Table A.2-3 a two-way ANOVA ($F [1, 44] = 0.02204$; $p = 0.08827$) indicated no statistically significant interaction between drug treatment and lifestyle intervention. Furthermore, no main factor differences were observed. With regards to MDA (Lipid peroxidation) data in Table A.2-3 a two-way ANOVA ($F [1, 36] = 2.394$; $p = 0.1305$) indicated no statistically significant interaction between drug treatment and lifestyle intervention. Furthermore, no main factor differences were observed. For SOD data in in Table A.2-3 a two-way ANOVA ($F [1, 35] = 16.96$; $p = 0.0002$; non-parametric analysis $\chi_1^2 = 11.053$; $p < 0.001$) indicated a statistically significant interaction between drug treatment and lifestyle intervention regarding percentage inhibition by SOD in the hippocampus on PostND61 in all treatment groups ($p < 0.0001$; $p < 0.001$; $p < 0.01$ respectively) as compared to the vehicle plus sedentary control group.

The data in Table A.2-3 suggest that pre-pubertal exercise, fluoxetine or the combination of exercise plus fluoxetine has no lasting effects on plasma corticosterone levels. In support of our findings it has been reported that, following chronic exercise, the plasma levels of corticosterone return to baseline (Marais, Stein & Daniels 2009, Soya et al. 2007, Ferreira et al. 2008) or are not even affected at all, as has been found with low intensity exercise (Soya et al. 2007). Some studies also reported elevated levels of corticosterone in healthy exercised rodents compared to controls (Zheng et al. 2006, Contarteze et al. 2008). The FSL, as a genetic animal model of depression, display increased plasma levels of corticosterone (Heim, Binder 2012) and although it is conceivable that fluoxetine could reduce plasma corticosterone, the current study did not demonstrate this in either of the fluoxetine-treated groups. The anti-depressive-like effects observed in our experiments are therefore likely independent of the HPA-axis. Furthermore, no significant differences between all treatment groups were observed in the levels of hippocampal lipid peroxidation (MDA in nmol/mg) suggesting that none of the interventions affected

hippocampal oxidative damage in these animals. Even though oxidative stress increases at the onset of exercise, adaptation occurs during chronic exercise, so that free radical concentration, protein or lipid damage in rat brain tissue is not enhanced following chronic exercise (Marais, Stein & Daniels 2009, Cechetti et al. 2007). Furthermore the increase in lipid peroxidation seems to be intensity dependent. High intensity exercise may lead to an increase in free radicals to such an extent that it could overwhelm anti-oxidant defences, whereas low intensity may even reduce oxidative stress to decrease levels of MDA (Cooper et al. 2002). Furthermore we did not observe any differences in hippocampal MDA in any of the fluoxetine treated groups, although studies in humans have found fluoxetine to decrease levels of MDA (Bilici et al. 2001). However, increased SOD, as found in all treatment groups, may indicate an increased anti-oxidant capacity following SSRI treatment (Bilici et al. 2001, Khanzode et al. 2003), or may result from an upregulation in anti-oxidants and pro-survival genes in response to increased lipid peroxidation, similar to what had been reported in other studies (Marais, Stein & Daniels 2009). That said, findings on disturbances in SOD activity are generally found in depressed patients, suggesting that SOD plays an important role in preventing oxidative damage (Kotan et al. 2011), as well as exert antidepressant-like properties in rats (Ferreira et al. 2008).

ADDENDUM B

CONGRESS PROCEEDINGS

In this section I present the abstract of data presented at a national congress in 2015.

Congress Proceedings

The results of the current study were presented as a podium presentation for the Young Scientist competition of the South African Society for Basic and Clinical Pharmacology 2015, held in Johannesburg. **The student as first and presenting author won the second prize in the category "Basic Pharmacology"**.

J.C. Schoeman, S.F. Steyn, B.H. Harvey, C.B. Brink. Pre-adolescent exposure to fluoxetine and/or exercise on depressive-like behaviour in stress-sensitive rats. The annual congress of the South African Society of Basic and Clinical Pharmacology in conjunction with Toxicology SA, Wits University, Johannesburg (31 August – 02 September 2015).

Abstract:

Background: Juvenile depression is a major concern worldwide with only the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and escitalopram approved for treatment. The effects of early-life exposure to SSRIs on neurodevelopment and subsequent lasting effects is not well understood. Exercise positively affects neuroplasticity, rendering exercise a potential augmentation strategy for drug therapy in juvenile depression. The current study investigated long-lasting effects of juvenile fluoxetine treatment and the potential role of exercise as treatment augmentation strategy in stress sensitive rats.

Materials and methods: Male Flinders Sensitive Line (FSL) rats (n = 12 per group) received either fluoxetine (5 mg/kg/day or 10 mg/kg/day subcutaneous) or vehicle control from postnatal day 21 (PostND21) to PostND34 (pre-adolescence), with or without simultaneous exposure to no, low or moderate intensity exercise (ethics approval no. NWU-00148-14-A5). Thereafter rats were housed normally and subjected to the open field and the forced swim tests on PostND35 or PostND60 (early adulthood) to assess locomotor activity and depressive-like behaviour, respectively.

Results: On PostND35, 5 mg/kg/day fluoxetine, but not 10 mg/kg/day, significantly decreased immobility vs. vehicle control. On PostND60 neither dose of fluoxetine altered immobility vs. vehicle control, whereas 5 and 10 mg/kg/day fluoxetine significantly decreased locomotor activity. Low intensity exercise alone showed a trend towards decreased immobility on PostND35. Data on the combined effect of fluoxetine and exercise are currently underway.

Conclusion: Chronic pre-adolescent administration of fluoxetine at low but not high dose induces an antidepressant response on PND35, although this response does not persist into adulthood. However, locomotor activity is reduced by both doses of fluoxetine, potentially masking its antidepressant effects. Preliminary data suggests that low intensity exercise alone does not exert significant effects on depressive-like behaviour. It remains to be seen from current studies whether exercise may augment fluoxetine treatment, and whether any such effects will persist into adulthood.

ADDENDUM C

GUIDELINES FOR AUTHORS

In this section I present the instructions given to the author for preperation and submission of the research article manuscript presented in chapter 3 of this dissertation.

The guidelines for the preparation of the article manuscript is outlined on the journal website: http://www.journals.elsevier.com/behavioural-brain-research/, under "Guidelines for Authors".

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ANNEXURES

LIST OF ABBREVIATIONS

5HT: 5 hydroxitriptamine/serotonin

5HTT: Serotonin Transporter

AChE: Acetylcholine Esterase

ACTH: Adrenocorticotropic hormone

ADHD: Attention Deficit Hyperactivity Disorder

APA: American Psychiatric Association

BDNF: Brain-Derived Neurotrophic Factor

CRHR1: Corticotropin Releasing Hormone Receptor 1

DFP: Diisopropylfluorophosphate

DSM-5: Diagnostic and Statistical Manual 5

ELISA: Enzyme-linked Immonusorbent Assay

EPM: Elevated Plus Maze

EXE: Exercise

FDA: Food and Drug Association

FLX: Fluoxetine

FRL: Flinders Resistant Line rat

FSL: Flinders Sensitive Line rat

FST: Forced Swim Test

GBD: Global Burden of Disease

GD: Gestational Day

HPA-axis: Hypothalamic-pituitary-adrenal Axis

$HR_{MAX:}$ Maximal Heart Rat**e**

IDO: Indoleamine 2,3 Dioxygenase

IFN-α: Interferon-alpha

IL-1: Interleukin-1

IL-6: Interleukin-6

IL-10: Interleukin-10

LPO: Lipid Peroxidation

LPS: Lipopolysacharide

MAOI: Mono-amine oxidase inhibitor

MDA: Malondealdehyde

MDD: Major Depressive Disorder

MOD: Moderate intensity exercise

mRNA: Messenger Ribonucleic Acid

NAC: n-Acetylcisteine

NARI: Noradrenalin Reuptake Inhibitors

NMDA: n-Methyl-D-aspartate

OFT: Open Field Test

PostND: Postnatal day

REM: Rapid Eye Movement

ROS: Reactive Oxygenated Species

SAL: Saline

SED: Sedentary

SIR: Social Isolation Rearing

SNRI: Serotonin and Noradrenalin Reuptake Inhibitors

SOD: Superoxide Dismutase

SSRI: Selective Serotonin Reuptake Inhibitors

TCA: Tricyclic Antidepressants

TMB: Tetramethylbenzidine

TNF-α: Tumour Necrotic Factor-alpha

VEH: Vehicle

VO_{2MAX}: Maximal Oxygen Consumption

WHO: World Health Organization

WST: Water soluble tetrazolium, [3-(4-Idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate