The long-term effects of fluoxetine on stress-related behaviour and acute monoaminergic stress response in stress sensitive rats

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Abstract:

Fluoxetine and escitalopram are the only antidepressants approved by the Food and Drug Administration of the United States of America (FDA) for treatment of major depression in children and adolescents. Both drugs are selective serotonin reuptake inhibitors (SSRIs). In recent years there has been a growing concern over the long-term developmental effects of early-life exposure to SSRIs.

The current study employed male Flinders Sensitive Line (FSL) rats, a well described and validated translational model of depression, to investigate the long term effects of pre-pubertal fluoxetine exposure. First we examined the effect of such early-life exposure on the development of depressive-like behaviour, locomotor activity and anxiety-like behaviour as manifested in early adulthood. Next, the current study investigated the effect of pre-pubertal fluoxetine exposure on the acute monoaminergic stress response, as displayed later in life. Animals received either saline (vehicle control), or 10 mg/kg/day fluoxetine from postnatal day (ND+) 21 to ND+34 (pre-puberty). The treatment period was chosen to coincide with a developmental phase where the serotonergic system's neurodevelopment had been completed, yet the noradrenergic and dopaminergic systems had not, a scenario comparable to neurodevelopment in human adolescents. Both behavioural and *in vivo* intra-cerebral microdialysis experiments were conducted after ND+60 (early adulthood).

On ND+60 rats allocated to behavioural experiments were evaluated for depressive-like behaviour in the forced swim test (FST), locomotor activity in the open field test (OFT), and anxiety-like behaviour in the OFT. Corticosterone concentrations were shown to be significantly higher in male FSL rats exposed to a 10 minute forced swim stress when compared to male FSL rats not exposed to a forced swim stress on ND+60. In the microdialysis experiments the rats were exposed to an acute 10 minute forced swim stress and the concentrations of the monoamines and their metabolites were measured before, during, and after the acute stressor.

Relative to saline-treated (control) rats, fluoxetine-treated FSL rats did not show long-term changes in immobility in the FST (i.e. no anti-depressant-like activity) on ND+60. Like-wise anxiety-like behaviour in the OFT did not change. However, a significant decrease in locomotor activity was observed in fluoxetine-treated FSL rats compared to saline-treated (control) rats. These data suggest that a long-lasting anti-depressant-like effect of fluoxetine may be masked by the effect on locomotor activity. With measurements from the microdialysis experiments a significant attenuation of the noradrenergic stress response was

observed in fluoxetine-treated rats compared to saline controls. A similar picture was observed for 5-hydroxyindole-3-acetic acid (5-HIAA), a metabolite of serotonin (5-HT), although the latter was not statistically significant. At baseline, before the stressor, significant increase in dopamine (DA) levels were observed in fluoxetine treated rats when compared to saline controls, suggesting that enhanced dopamine neurotransmission may comprise a long-term effect of pre-pubertal fluoxetine treatment. There were no discernible differences in homovanilllic acid (HVA) concentrations between fluoxetine-treated rats and saline controls. In conclusion significant developmental effects of pre-pubertal fluoxetine exposure were observed later in life and these findings warrant further investigation.

Keywords: Depression**,** Neurodevelopment, Fluoxetine, Flinders Line Sensitive rat, Monoaminergic stress response, Depressive-like behaviour, Locomotor activity.

Opsomming:

Fluoksetien en essitalopram is die enigste antidepressante wat deur die “Voedsel en Geneesmiddel Administrasie (“Food and Drug Administration” - FDA) van die Verenigde State van Amerika goedgekeur is vir die behandeling van major depressie in kinders en adolosente. Beide hierdie middels is selektiewe serotonienheropname-inhibeerders (SSHI’s). In die laaste aantal jare was daar ’n toenemende kommer oor die langtermyn ontwikkelingseffekte van vroeë-lewe blootstelling aan SSHI’s.

Die huidige studie het gebruik gemaak van manlike Flinders Sensitiewe Lyn- (FSL-) rotte, ’n goed beskryfde genetiese translasie-model van depressie, om die langtermyn effekte van pre-puberteit fluoksetien-blootstelling te ondersoek. Eerstens het ons ondersoek ingestel om na die effek van sodanige vroeë-lewe blootstelling op die ontwikkeling van depressie-agtige gedrag, lokomotor aktiwiteit, en angs-agtige gedrag soos gemanifesteer in vroeë volwasenheid te kyk. Tweedens het die huidige studie ondersoek ingestel om na die effekte van pre-pubertale fluoksetien-blootstelling op die akute monoaminergiese stresrespons, soos vertoon later in die lewe te bestudeer. Diere het ‘n soutoplossing (draer-kontrole) of 10 mg/kg/dag fluoksetien vanaf postnatale dag (ND+) 21 tot ND+34 (pre-puberteit) ontvang. Die behandelingsperiode was gekies om ooreen te stem met ’n periode waar die serotonergiese stelsel se neuro-ontwikkeling voltooi is, maar waar die noradrenergiese en dopaminergiese stelsels s’n nog nie ten volle ontwikkel is nie - ’n scenario vergelykbaar met die neuro-ontwikkeling in die menslike adolosent. Beide gedrag en *in vivo* intra-serebrale mikrodialise-eksperimente was uitgevoer na ND+60 (vroeë volwassenheid).

Op ND+60 is rotte wat geallokeer was vir gedragseksperimente geëvalueer vir depressie-agtige gedrag in die geforseerde swemtoets (GST), lokomotoraktiwiteit in die oopveldtoets (OVT), en angs-agtige gedrag in die OVT. In die mikrodialise-eksperimente was rotte blootgestel aan ’n akute 10-minute geforseerde swem stres en die konsentrasies van die monoamiene en hul metaboliete was gemeet voor, tydens en na die akute stressor.

Relatief tot die soutoplossing-behandelde (kontrole) rotte, het fluoksetien-behandelde FSL-rotte nie langtermyn veranderinge in immobiliteit in die GST op ND+60 vertoon nie (d.w.s. geen anti-depressant-agtige aktiwiteit nie). Soortgelyk het angs-agtige gedrag in die OVT nie verander nie. ’n Beduidende vermindering in lokomotoraktiwiteit was egter waargeneem in die fluoksetien-behandelde FSL-rotte, relatief tot die draer-behandelde (kontrole) rotte. ’n Soortgelyke beeld was waargeneem vir 5-hidroksie-indool-3-asynsuur (5-HIAA), ’n metaboliet van serotonien, alhoewel die laasgenoemde nie statisties betekenisvol was nie.

By die basislyn, voor die stressor, was ’n beduidende verhoging in dopamienkonsentrasies waargeneem in fluoksetien-behandelde rotte in vergelyking met soutoplossing-behandelde kontroles, wat suggereer dat verhoogde dopaminergiese neurotransmissie een van die langtermyneffekte van pre-puberteit-fluoksetien-behandeling mag wees. Daar was geen onderskeibare verskille in die homovaniliensuur (HVA)-konsentrasies tussen fluoksetien-behandelde rotte en soutoplossing-kontroles nie. Ten slotte was beduidende ontwikkelingseffekte van pre-pubertale fluoksetien-blootstelling later in die lewe waargeneem en hierdie bevindinge regverdig verdere ondersoek.

Sleutelwoorde: Depressie**,** Neuro-ontwikkeling, Fluoksetien, Flinders Lyn Sensitiewe-rot, Monoaminergiese stresrespons, Depressief-agtige gedrag, Lokomotor aktiwiteit.

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List of abbreviations

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APA: American psychiatric association

BDNF: brain derived neurotrophic factor

Ca^{2+}: calcium ions

CA: catecholamine

CES-D: Centre for epidemiological studies depression scale

cGMP: cyclic guanosine monophosphate

DA: dopamine

DAT: dopamine transporter

DNA: deoxyribonucleic acid

DSM: diagnostic and statistical manual

ECF: extracellular fluid

FC: frontal cortex

FDA: Federal Drug Administration

FSL: Flinders sensitive line

FST: **f**orced swim test

HPA: hypothalamic-pituitary-adrenal

HPLC: high performance liquid chromatography

HVA: homovanillic acid

IL: interleukin

i.p.: intra peritoneal

LPS: lipopolysaccharide

MDD: major depressive disorder

mGluR: metabotropic glutamate receptor

MHPG: 3-methoxy-4-hydroxyphenylglycol

ND+: postnatal day

ND-: prenatal day

NE: norepinephrine

NET: norepinephrine transporter

NIMH: National Institute of Mental Health

NMDA: 3-methoxy-4-hydroxyphenylglycol

NO: nitric oxide

NOS: nitric oxide synthase

OCT: organic cation transporter

OFT: open field test

PDE5: phosphodiesterase 5

PFC: prefrontal cortex

RNA: ribonucleic acid

RNS: reactive nitrogen species

ROS: reactive oxygen species

rpm: revolutions per minute

s.c.: subcutaneous

SERT: serotonin transporter

SSRI: selective serotonin reuptake inhibitor

TNFα: tumour necrosis factor α

TrkB: tyrosine kinase B

VMA: vanillylmandelic acid

WHO: World Health Organisation

Chapter 1: Introduction

1.1 Dissertation Approach and Layout

This dissertation is presented in an article format. The essential data have been prepared for publication in a selected scientific journal. Additional data not included in the article, but no less important in understanding the study as a whole, is presented in Addendum A. 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 the 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

- Chapter 4: 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 a mood disorder affecting a significant proportion of the global population, in fact, according to the World Health Organisation; it is the leading cause of disability world-wide (Marcus *et al*., 2012). More than 350 million individuals suffer from MDD (Marcus *et al*., 2012). The epidemiological data on MDD in the young reflect a similar MDD prevalence in adults and adolescents (Birmaher *et al*., 1996; Costello *et al*., 2006 & Kessler *et al*., 2010; Rhode *et al*., 2013). In pre-puberty the prevalence of MDD is between 0 to 2% (Eggar & Angold, 2006). Between mid and late adolescence the prevalence increases to 4-5% (Thapar *et al*., 2012). Some concern has developed due to various findings in studies which suggest that MDD is becoming a greater problem in the young. An example of such a finding is that of Zito and colleagues (2003) who showed that an increased amount of prescriptions for antidepressants in children and adolescents are being issued. An increased suicide rate has also been observed in adolescents of the United States of America over a period of 40 years between 1950 and 1990 (Costello *et al*., 2006).

Only fluoxetine and escitalopram use has been approved by the FDA in children and adolescents for MDD (Soutullo & Figueroa-Quintana, 2013). Both agents are selective serotonin reuptake inhibitors (SSRIs) (Beldessarini, 2006). According to Mulder and colleagues (2011) several studies have shown that SSRIs cause foetal developmental changes when used for MDD in pregnant mothers. They also state however that 5 year follow-up studies show no lasting negative effects on children exposed to these antidepressants *in utero* with regard to cognition, temperament, internalising and externalising behaviours. SSRIs have also been found to cause developmental changes in animal studies. Prenatal exposure to serotonin transporter protein (SERT) inhibiting agents such as SSRIs, but not norepinephrine transporter (NET) inhibitors, were shown to produce undesirable behavioural outcomes in mice later in life (Ansorge *et al*., 2008). SSRIs like fluoxetine and escitalopram increase synaptic concentrations of serotonin (5-HT) by preventing its re-uptake via SERT (Beldessarini, 2006). SSRIs prescribed to children and adolescents with MDD may therefore have profound consequences in terms of neurodevelopment. According to Murrin and colleagues (2007) these drugs may cause developmental alterations to both serotonergic and non-serotonergic pathways.

Stress has been shown to cause alterations in various systems implicated in MDD including monoaminergic neurotransmission, inflammation (due to the effects of the hypothalamic-pituitary-adrenal (HPA) axis on immune system functioning) as well as neurogenesis and neuroplasticity (Ehlert *et al*., 2001; Leonard, 2001; Schiepers *et al*., 2005). Increases in serotonergic, noradrenergic and dopaminergic functioning has been described in previous studies as well (Kvetnansky *et al*., 2009; Rueter *et al*., 1997).

The current study investigated the possible developmental effects of fluoxetine (one of two antidepressants approved by the FDA for use in children with MDD) in a genetic rat model of depression, the Flinders Sensitive Line (FSL) rat. Firstly, we explored changes in the depressive-like behaviour, locomotor activity and anxiety-like behaviour of FSL rats (treated during pre-puberty) relative to control animals which received saline injections (also during pre-puberty). Changes to neurobiological stress mechanisms which may be produced via early-life administration of fluoxetine were also examined. Here we made use of microdialysis to investigate monoaminergic release and reuptake as well as enzymatic turnover in awake, freely moving rats before, during and after exposure to an acute stressor.

1.3 Study Objectives

1.3.1 Primary objective

This study investigated the later-life effects of chronic fluoxetine administration during pre-puberty (i.e. ND+21 to ND+34), in stress-sensitive rats as determined in early adulthood (ND+60). Endpoints assessed included:

- Monoaminergic stress-responses by measuring *in vivo* prefronto-cortical monoamine concentrations via microdialysis before, during and after an acute swim stress
- Depressive-like, locomotor and anxiety-like behaviour as assessed in the forced swim test (FST) and open field test (OFT)

1.3.2 Secondary Objective

The secondary objective of the study was to implement and validate the *in vivo* intracerebral microdialysis technique with high performance liquid chromatography (HPLC) measurement of brain monoamines in rats, followed by its application in a translational animal model of depression (the FSL rat).

1.4 Study Layout

Figure 1-1 depicts the layout of the study as a whole.

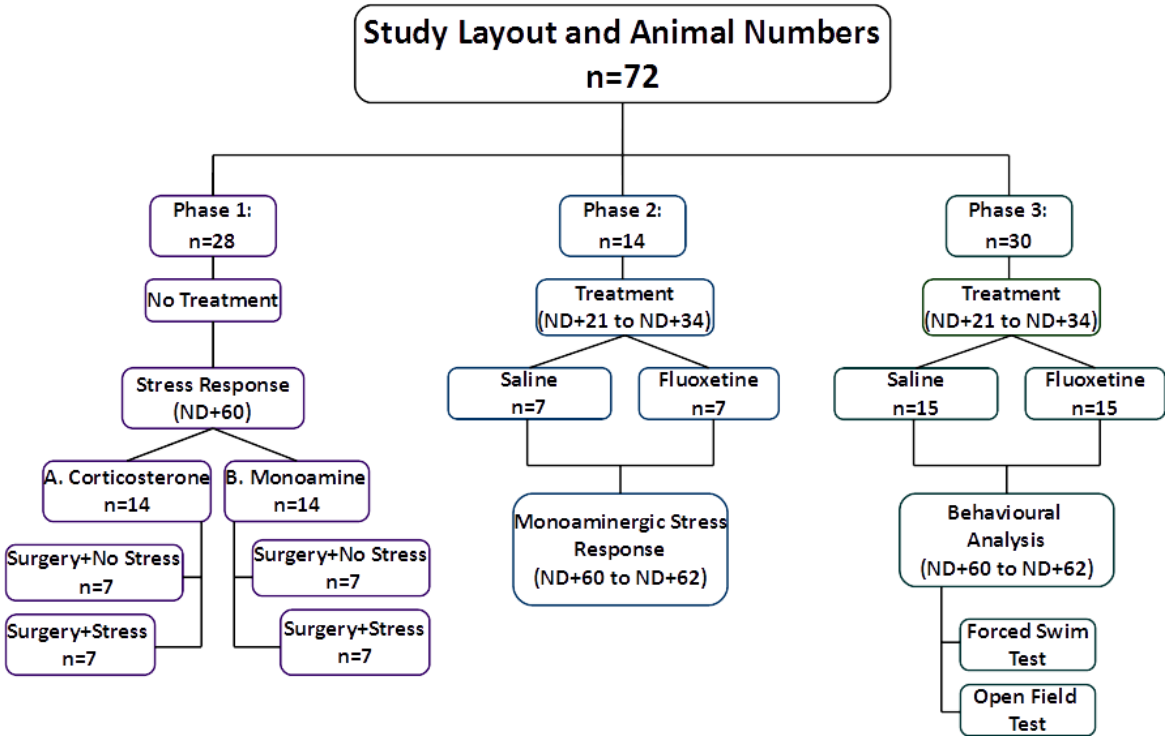

Figure 0-1: Graphic representation of the study layout. ND+ = Postnatal day; n= number of subjects.

The study was divided into three distinct phases which will now be described in some detail. All studies were conducted in FSL rats.

1.4.1 Phase 1: Stress Response

1.4.1.1 Phase 1A: Corticosterone Response

Acute swim stress has been successfully employed as stressor in previous studies (Purdy *et al*., 1991; Schwartz *et al*., 1987). It was now necessary to verify that swim stress is an effective physiological stressor under our experimental conditions. Briefly, as seen in Figures 1-1 and 1-2, guide cannulas were placed in the prefrontal cortex (PFC) of FSL rats on ND+57. Between ND+59 and ND+61 untreated rats were placed in an airtight halothane enclosure until immobile to simulate the probe placement procedures as used in phase 1B and phase 2 (described in 1.4.1.2 and 1.4.2). After overnight recovery, the rats were exposed to either 0 or 10 minute forced swimming in an enclosed cylinder (acute stressor). The rats were decapitated, trunk blood was collected in heparanised blood tubes, and corticosterone concentrations were measured.

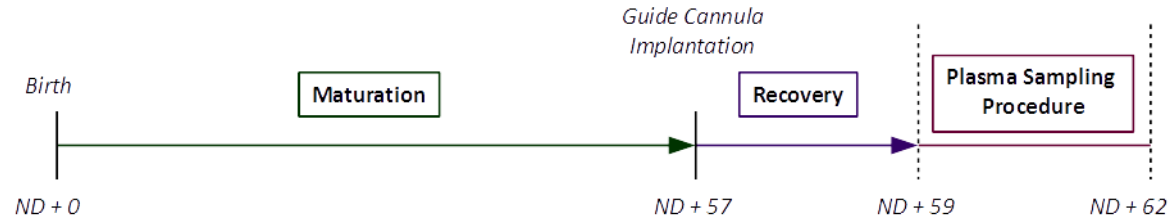

Figure 0-2: Schematic representation of the corticosterone stress response study. ND+ = Postnatal day.

1.4.1.2 Phase 1B: Monoaminergic Stress Response

In another pilot study, depicted in Figures 1-1 and 1-3, it was attempted to demonstrate a significant acute monoaminergic response to forced swim stress, as measured in dialysate from the PFC of FSL rats. For this purpose an experiment was designed similar to the one described above, but now measuring monoamine concentrations in samples collected by means of microdialysis. One group was exposed to the acute swim whereas the other group was not. The subjects in this experiment were also not exposed to any pharmacological treatment as described for consecutive experiments below.

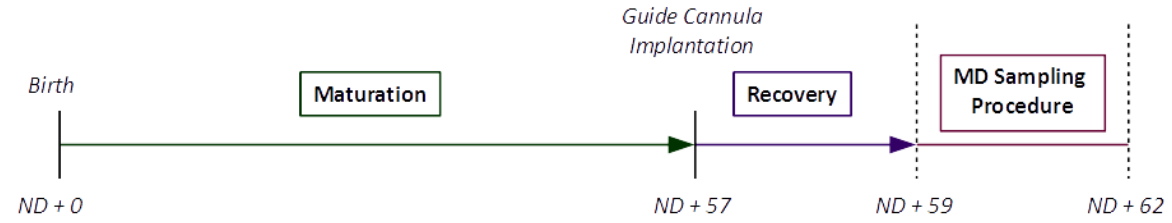

Figure 0-3: Schematic representation of the monoaminergic stress response study. ND+ = Postnatal day; MD= Microdialysis.

1.4.2 Phase 2: Effects of Fluoxetine on Monoaminergic Stress Response

In this phase of the study, depicted in Figures 1-1 and 1-4, FSL rats were treated with saline (as vehicle control) or fluoxetine (10mg/kg/day) from ND+21 to ND+34. Thereafter the rats underwent the same procedures, including the microdialysis and measurement of monoaminergic stress response, as described in Phase 1B (see 1.4.1.2).

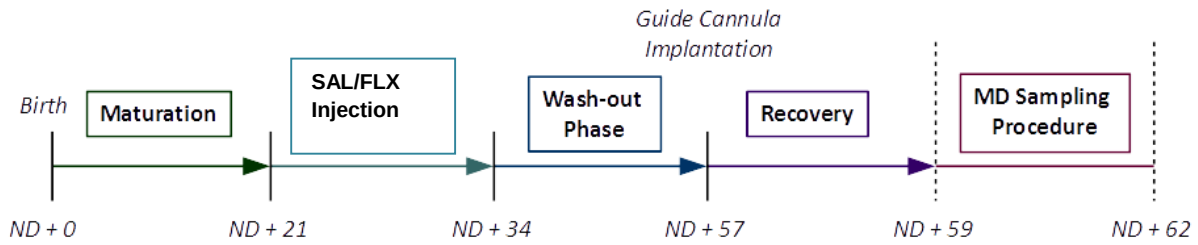

Figure 0-4: Schematic representation of the second phase of study. ND+ = Postnatal day; MD = Microdialysis; FLX= Fluoxetine; SAL=Saline.

1.4.3 Phase 3: Effects of Fluoxetine on Depressive-like Behaviour

In this phase of the study rats were treated with saline (as vehicle control) or fluoxetine (10 mg/kg/day) from ND+21 to ND+34, similar to that described in Phase 2 (see 1.4.2). Thereafter rats were housed normally until ND+60 (representing a drug washout period). On ND+60 these animals underwent behavioural analyses, as depicted in Figures 1-1 and 1-5, measuring depressive-like behaviour in the forced swim test (see 3.2.3.1 and A.1.4.2), as well as anxiety-like behaviour and locomotor activity in the open field test (see 3.2.3.2 and A.1.4.1).

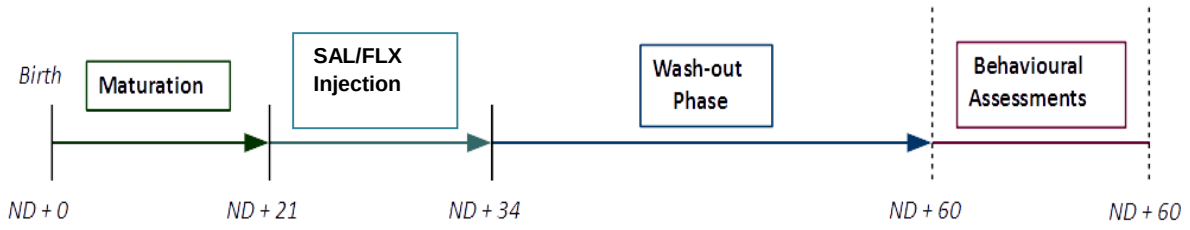

Figure 0-5: Schematic representation of the third phase of study. ND+ = Postnatal day; FLX= Fluoxetine; SAL=Saline.

1.5 Hypothesis and Expected Results

My working hypothesis was that administration of fluoxetine (SSRI) during pre-puberty in FSL rats would protect against the development of depressive-like symptoms during early adulthood. Firstly, I postulated that the pro-serotonergic effects of fluoxetine (administered after the completion of serotonergic development) between ND+21 to ND+34, would induce beneficial changes with regard to the development of depressive-like behaviour in later life. The rapid development of the serotonergic system has been postulated to suggest a role in the development of other neurotransmitter systems (for a more detailed discussion see 2.10). I hoped to exploit this by inducing changes within the noradrenergic system (neurodevelopment only completed on ND+35) and the dopaminergic system (neurodevelopment only completed on ND+60). I expected to see observable differences between treatment groups with regard to depressive-like behaviour and monoamine concentrations within microdialysis samples.

1.6 Ethical Approval

Ethical approval for this study was obtained from the Ethics Committee of North-West University. All the procedures involving the experimental animals in this study were conducted according to the National Institute of Health guidelines for the care and use of laboratory animals.

Approval numbers:

- NWU-00045-10-5S: Behavioural component of the study
- NWU-0028-08-A5: Microdialysis component of the study

Chapter 2: Literature review

Major depressive disorder is a serious neuropsychiatric disease affecting the health and well-being of many individuals. In particular, the diagnosis and associated treatment of MDD and anxiety-related disorders have escalated in children and adolescents during the last decades. In this chapter various aspects MDD will be elucidated with specific emphasis placed on its impact on children and adolescents. First, the global and local epidemiology will be outlined, followed by a discussion of the clinical signs, symptoms, and diagnosis, as well as the neurobiological aetiology & hypotheses. Thereafter treatment options will be discussed, as well as current animal models of depression. More in-depth discussions will follow on the role of the monoaminergic system in MDD and neurodevelopment. The chapter will conclude with a synopsis of the literature findings.

2.1 Epidemiology

Major depression is a mood disorder affecting a significant proportion of the global population, including people of all ages, ethnicities, and socio-economic backgrounds. According to the World Health Organisation (WHO) (Marcus *et al*., 2012) it is the leading cause of disability world-wide in terms of time spent suffering from the disease, with more than 350 million individuals suffering from the disorder at any particular point in time (Marcus *et al*., 2012). The WHO also reports that females have a 50% greater burden of disease than males (Marcus *et al*., 2012). It has been reported that 2-5% of the world population is affected by MDD and that the disease has a lifetime prevalence of 15% in the United States of America (Bylund & Reed, 2007). A lifetime prevalence of 9.8% was reported for South Africa, which is lower than the lifetime prevalence of MDD in the United States of America, but higher than other African countries such as Nigeria (Tomlinson *et al*., 2009).

Various authors (Birmaher *et al*., 1996; Costello *et al*., 2006 & Kessler *et al*., 2010) have reported that the prevalence of MDD in adulthood is similar to that seen in late adolescence. This has been confirmed by Rohde and colleagues (2013) in a recent longitudinal study of MDD. The prevalence of depression in pre-pubertal children varies between 0-2 % with no marked difference between males and females (Eggar & Angold, 2006). According to Thapar and colleagues (2012) the prevalence in mid to late adolescence rises to 4-5%, which is comparable to that of adults. It is also interesting to note that it is at this age that the prevalence in females rises disproportionately to mirror the adult prevalences of MDD (Maughan *et al*., 2013).

According to Costello and colleagues (2006) the impression exists that MDD is becoming an increasing problem in children and adolescents and has reached epidemic proportions. Indeed, there are 4 primary sources of data which, when viewed collectively, leads to this conclusion (Costello *et al*., 2006). The first is the finding by Zito and colleagues (2003) that an increasing number of prescriptions for antidepressants are being written for children and adolescents. Secondly, an increase in the suicide rate of adolescents was observed in the United States of America between 1950 and 1990 (Costello *et al*., 2006). Thirdly, in three British birth cohorts (1974, 1986, and 1999) increased "emotional problems" (anxiety and depression) were reported among participants at age 15 to 16 (Costello *et al.,* 2006). Lastly, various epidemiological studies were conducted of retrospective recall of depression symptoms in successive birth cohorts of adults (Costello *et al*., 2006). However, a meta-analysis of the available epidemiological data by Costello and colleagues (2006) concluded that the apparent increase of MDD in these age groups is more likely the result of an increasing awareness of a disorder which has been, up to now, underdiagnosed in children rather than an epidemic of MDD in this age group.

Reasons for the observed and reported rise in antidepressant prescriptions for children and adolescents remain to be illuminated. It is of particular importance that we better understand the long-term effects of such juvenile interventions, even more so when one considers that neurodevelopment is incomplete in these individuals and that it may therefore be influenced by the introduction of any agent that alters neurochemistry.

2.2 Signs and Symptoms of MDD

According to the National Institute of Mental Health (NIMH) of the United States of America and the WHO (Marcus *et al*., 2012), symptoms of depression include feelings of hopelessness, helplessness, and worthlessness. These individuals are also subject to pessimism, anxiety, sadness, anhedonia, and irritability. They display cognitive impairment characterised by lack of concentration, memory impairment, and an impaired ability to make decisions (NIMH; Marazziti *et al*., 2010). Sufferers experience sleep and dietary disturbances and may over- or under-indulge in these activities. Feelings of fatigue and low energy levels are also experienced. Various systemic disturbances such as headaches or gastrointestinal symptoms which do not respond to conventional treatment can also be a sign of MDD. The most important manifestations of MDD are suicidal ideation and, in severe cases, suicide attempts (NIMH; Marcus *et al*., 2012). In children and adolescents, the overall presentation and clinical symptoms of MDD are similar to adult depression. In addition, there is also a possibility that depression may, in these age groups, prevent the development of a healthy personality and hence give rise to personality disorders (Bylund & Reed 2007).

Various neuroanatomical changes are associated with MDD. These changes have been observed in specific brain regions and include the PFC and subdivisions (dorsolateral PFC, orbitofrontal cortex, medial PFC, and anterior cingulate cortex), amygdala, hippocampus, raphe nucleus, and locus coeruleus (Hercher *et al*., 2009). The changes described above may either be aetiological in nature or reflect compensatory adaptive mechanisms, which occur due to the disease processes (Mayberg, 2003). The PFC and hippocampal areas have been implicated in the neuropathology of MDD in numerous studies, and hence their roles are well described (Hercher *et al*., 2009; Mayberg, 2003). Accordingly, the structural and neurochemical changes associated with MDD in these areas are described in more depth below.

Analysis of morphological and morphometric data of the hippocampus, collected from depressed patients, show structural changes in terms of volume reduction, alterations of gray matter, and neuropil reductions (Sheline, 2000; Stockmeier *et al*., 2004). It has been reported that patients with longer durations of MDD have greater left hippocampal volume reductions than patients with a shorter duration of illness (MacMaster & Kusumakar, 2004; Shah *et al*., 1998). A recent meta-analysis of volumetric changes associated with MDD found no difference in the loss of hippocampal volume between the right and left hemispheres (McKinnon *et al*., 2009). This is in agreement with various other studies that show no difference between left and right hippocampal volume reduction (Campbell *et al*., 2004; Videbech & Ravnkilde, 2004). Jacobs (2002) postulated that changes in adult hippocampal neurogenesis could be responsible for these structural alterations. This hypothesis is based on evidence that antidepressant drugs stimulate proliferation of progenitor cells found in the hippocampus (Perera *et al*., 2007). It is interesting to note that the period of onset of the antidepressive effect of commonly used antidepressants parallels the maturation period of neurons newly synthesised in the mature hippocampus (Dranovsky & Hen, 2006). There is therefore considerable evidence which point to hippocampal involvement in depression.

In the PFC MDD-induced changes include blood flow alterations, volume reduction of both gray and white matter, altered glucose metabolism, and widening of the sulci (Hercher *et al*., 2009). Furthermore, changes in the density and quantity of the glial cells have been reported in sufferers of MDD (Hercher *et al*., 2009). The reduction seen in gray matter occurs notably in the left hemisphere, in particular the subgenual anterior cingulate cortex, and this reduction appears to be more severe in individuals with a family history of MDD and in individuals with chronic or recurrent MDD (Price *et al*., 2012). Oligodendrocytes are the cells responsible for the formation of the myelin sheaths (neurons with axons covered by these

sheaths are known as white matter) within the central nervous system (Ransom, 2009). There is a decrease in the quantity and density of oligodendrocytes in patients suffering from MDD (Uranova *et al*., 2004). Steiner and colleagues (2008) showed that there is an increased density in human leukocyte antigen-DR (a marker for neuroinflammation and neurodegeneration), labelled microglia in several brain regions in patients with MDD who had committed suicide. These findings emphasise the importance of the potential role that glial cells may play in the pathogenesis of MDD.

Positron emission tomography and single photon emission tomography studies have shown abnormalities in frontal blood flow and glucose metabolism (Mayberg, 2003; Monkul *et al*., 2012). These changes have been reported to be more severe in the anterior cingulate cortex and PFC in the left cerebral hemisphere (Wilner *et al*., 2013). N-acetyl-aspartic acid levels, a marker for neurodegeneration, become more decreased within the medial PFC of paediatric MDD patients the longer the disease persists (Olvera *et al*., 2010).

2.3 Diagnosis of MDD

A major depressive episode is diagnosed based on the criteria listed in the Diagnostic and Statistical Manual (DSM)-5 developed by the American Psychiatric Association (APA, 2013a). These criteria are essentially the same as those used in the classification system which preceded the DSM-5 known as the DSM-IV (APA, 2013a). The first major change from the DSM-IV is that the DSM-5 allows for a diagnosis of major depressive episode to be made in the presence of 3 manic symptoms if they are of such a nature that a diagnosis of manic episode cannot be made. The diagnosis of a major depressive episode is then characterised as having mixed features (APA, 2013b). The second major change from the DSM-IV in the DSM-5 is that a diagnosis of major depressive episode can now be made when a loved one has been lost. This is due to the recognition that bereavement appears to last for 1-2 years after such an event and not only 2 months as stated in the DSM-IV. Secondly the signs and symptoms associated with a bereavement-related major depressive episode are the same as those seen in “classical” major depressive episodes. The authors now view bereavement as one of many stressors which may cause a major depressive episode (APA, 2013b). The last change is that criterion C from the DSM-IV is now listed as criterion B (APA, 2013b). Five of the criteria listed below have to be present over a single fortnight with a significant impact on the normal functioning of the individual. Furthermore, at least one of the first two criteria (along with a minimum of 4 additional criteria) needs to be present for this diagnosis to be made. The essential and additional criteria are listed together under A in the DSM but are separated here for clarity (APA, 2000).

2.3.1 Essential criteria

- depressed mood for the greatest part of each day (subjectively reported or observed by another individual)
- loss of interest or pleasure in daily activities on most days (subjectively reported or observed by another individual)

2.3.2 Additional criteria

- significant weight loss or gain or appetite changes (increase or decrease in appetite nearly every day or, in children, failing to achieve expected weight gains
- altered sleep patterns (insomnia or hypersomnia) on most days
- frequent signs and symptoms of psychomotor agitation or retardation
- lack of energy or fatigue on most days
- feelings of worthlessness or excessive or inappropriate guilt (possibly delusional) on most days which is not merely self-reproachful or due to feeling guilty about not being healthy
- difficulty in ability to think or concentrate or indecisiveness on most days (self-reported or observed by someone else)
- continued contemplation of death (not just fear of dying), recurrent suicidal ideation in the presence or absence of concrete plans to commit suicide, or a suicide attempt

2.3.3 Exclusion criteria

- The effects of medication or drugs of abuse or manifestations of another disease process (e.g. hypothyroidism) are not responsible for the diagnosis.

2.4 Aetiology of MDD

2.4.1Genetic Factors

MDD appears to be a complex disease with intricate genetic properties. Twin studies (both monozygotic and heterozygotic) indicate that MDD has a heritability rate of 37%, which is lower than the heritability rates of schizophrenia and bipolar disorder (Belmaker & Agam, 2008). It seems that the earlier the age of onset of MDD, the more severe the disease. The recurrent forms of the disease are associated with greater heritability (Kendler *et al*., 1999). No single chromosomal abnormality has been identified in every familial MDD study (Belmaker & Agam, 2008). A polymorphic variant of serotonin-transporter-linked polymorphic region has been implicated in MDD and is also associated with a more anxious and pessimistic personality type (Caspi *et al*., 2003; Lesch, 2002). Epigenetic changes may also

play a role in the development of MDD as demonstrated by Weaver and colleagues (2004) in animal studies using rats where they found that maternal pup grooming and nursing behaviour results in altered fearfulness levels and responses to stress later in life. They were also able to demonstrate that these changes were associated with altered deoxyribonucleic acid (DNA) methylation (Weaver *et al*., 2004). Another example of this phenomenon was provided by Melas and colleagues (2011) who managed to reverse hypermethylation of the p11 gene (a gene associated with depression) promotor in FSL rats, a genetic rat model of depression, with the clinically used SSRI class antidepressant, escitalopram.

Collectively, there is evidence to suggest a strong correlation between genetic susceptibility and MDD. As described in further detail below we will make use of the FSL rat in this study which is a validated genetic rat model of depression.

2.4.2 Monoamine-Deficiency Hypothesis

Abnormalities in monoamine neurotransmission have been proposed as a leading candidate for explaining the neurobiological basis of MDD for more than 40 years (Gardner & Boles, 2011). The effect of most clinically effective antidepressant drugs is to increase the synaptic concentrations of norepinephrine (NE) and 5-HT, suggesting that synaptic monoamine deficiency is strongly associated with depression (Belmaker & Agam, 2008). Agents originally developed for the treatments of Parkinson's disease (such as buproprion, a DA reuptake inhibitor, and pramipexole, a direct DA receptor agonist) have proven to be effective in the treatment of depression as well (Gershon *et al*., 2007). The major criticism of the monoamine deficiency hypothesis is that, despite the immediate increase in synaptic concentrations of monoamines as a result of antidepressants, the antidepressant effect usually takes between two to six weeks to manifest (Racagni & Popoli, 2008). Furthermore, approximately a third of MDD sufferers do not respond to conventional antidepressant treatment (Mann, 2005). A third problem with this hypothesis is that drugs such as amphetamine and cocaine increase the synaptic monoamines but are not clinically effective as antidepressants (Rang *et al*., 2003). Numerous studies have failed to demonstrate decreased monoamine metabolite levels in various body fluids, as well as decreased monoamine levels in brain tissue of MDD sufferers (Belmaker & Agam, 2008). These findings suggest that the aetiology is more complex than a simple deficiency of monoamines in the synaptic cleft. Rather it has been suggested that elevated monoamine levels have secondary effects on receptor concentration and related changes in cellular plasticity, resulting in altered neurotransmission (Racagni & Popoli, 2008). This typically takes several days to weeks to develop and presumably underlies the eventual clinical manifestation of the antidepressant effect of antidepressants (Blier, 2003).

2.4.3 The Hypothalamic-Pituitary-Adrenal Axis Hyperactivity Hypothesis

The processes involved with normal HPA axis function will now be described (Belmaker & Agam, 2008). Cortical brain structures stimulate the hypothalamus to secrete corticotropin-releasing hormone due to stress (the concept of stress is discussed in greater depth in 2.9). This will, in turn, stimulate the corticotropin-releasing hormone receptors of the pituitary gland, which then stimulates the release of corticotropin. Corticotropin then circulates in the plasma and, upon reaching the adrenal cortex, causes the release of cortisol into the blood. A negative feedback loop will decrease the amount of corticotropin-releasing hormone released. This regulating mechanism is activated by cortisol binding to cortisol receptors on the hypothalamus. If this system is chronically hyperactive it will lead to elevated cortisol levels and a desensitisation of cortisol receptors (Belmaker & Agam, 2008).

The role of stress in the development of MDD appears to be rather complex with many factors playing a role. Features associated with HPA axis hyperactivity include modulation of monoaminergic neurotransmission, inflammation (due to the effects of the HPA axis on immune system functioning (discussed in 2.4.6)) as well as neurogenesis and neuroplasticity (Ehlert *et al*., 2001; Leonard, 2001; Schiepers *et al*., 2005). It has been reported that cortisol and corticotrophin releasing hormone levels are altered in various patients who suffer from MDD (Carroll *et al*., 2007; Holsboer, 2000; Merali *et al*., 2004). HPA axis over activity is generally associated with MDD patients (Carroll *et al*., 2007). Furthermore, various non-steroidal anti-inflammatory drugs hold promise as adjunctive therapy in patients suffering from treatment resistant MDD (Miller *et al*., 2009; Schlaepfer *et al*., 2012). Centrally acting non-steroidal anti-inflammatory drugs and minocycline (a tetracycline antibiotic agent with anti-inflammatory activity) have shown some efficacy in treatment of treatment resistant MDD (Molina-Hernandez *et al*., 2008; Akhondzadeh *et al*., 2009).

2.4.4 Neuroplasticity Hypothesis of Depression

Brain-derived neurotrophic factor (BDNF) has been shown to play a key role in various neurodevelopmental and neuromodulatory activities. It also has neuroprotective properties and its deficiency has been postulated to be an important factor in the development of MDD (Angelucci *et al*., 2005; Kozlovsky *et al*., 2007). BDNF has potent neurotrophic actions for serotonergic neurons when administered to the midbrain, whereas it induces a greater serotonergic fibre density when administered to the forebrain (Angelucci *et al*., 2005). BDNF also prevents neurotoxic damage to neurons when administered directly to the forebrain (Angelucci *et al*., 2005). Kozlovsky and colleagues (2007) showed that increased rat plasma corticosterone (cortisol equivalent in the rat) was associated with decreased hippocampal BDNF, especially in the cornu ammonis 1 and dentate gyrus subregions. They also reported

a decrease in tyrosine kinase B (TrkB) receptor, the BDNF receptor, in these areas and the frontal cortex (FC) under these conditions (Kozlovsky *et al*., 2007). BDNF concentrations and TrkB expression within the hippocampus and FC are decreased in brain tissue of suicide victims, collected post mortem (Dwivedi *et al*., 2003). BDNF levels are increased by all antidepressants (Duman & Monteggia, 2006). In addition, decreased levels of BDNF are associated with increased stress levels (Angelucci *et al*., 2005). All of these findings suggest that MDD is the result of a complex interplay of factors such as stress, neurodevelopment, and neuromodulation.

2.4.5 Glutamate Hypothesis of Depression

Dysfunction of the glutamatergic neurotransmitter system has been suggested to be involved in the development of MDD (Sanacora *et al*., 2012). In a recent review Sanacora and colleagues (2012) postulated the glutamate hypothesis of MDD, suggesting that it might account for various pre-clinical and clinical findings associated with the disease and provide an integrative framework for a more complete understanding of the pathophysiology of the disease. According to this working hypothesis elevated glutamatergic neurotransmission will result in MDD symptoms via over stimulation of the N-methyl-D-aspartate (NMDA) receptors.

Glutamate is an excitatory amino acid neurotransmitter and glutamatergic neurons form the major excitatory pathways in the brain (Sanacora *et al*., 2012). It plays an important role in various processes in the brain including learning, memory, and neuroplasticity via processes such as long-term potentiation and long-term depression (Krystal, 2007). Once glutamate has been released into the synaptic cleft it can bind to one of four types of post-synaptic receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), NMDA, kainate, and metabotropic glutamate receptors (mGluR). In addition it may undergo reuptake into the releasing neuron or it may be transported into the surrounding glial cells via excitatory amino acid transporters, where the molecule is enzymatically converted by glutamine synthetase to glutamine (Bloom, 2006). The latter may then be released into the extracellular fluid (ECF) and transported into the pre-synaptic neuron where it is enzymatically changed back to glutamate.

As seen in Figure 2-1 glutamate stimulation of NMDA receptors results in the creation of nitric-oxide (NO) via calcium ion (Ca^{2+}) mobilisation. This causes the activation of nitric-oxide synthase (NOS) which results in the formation of NO (Dhir & Kulkarni, 2011; Yamamoto *et al*., 2010). NO is an important second messenger and plays an important role in neuromodulation as evidenced by its role in various neurological processes such as neurotransmission, neuroplasticity, and changes in gene expression (Esplugues, 2002; Dhir

& Kulkarni, 2011). NO is able to play a role in all these various processes due to its unique ability to diffuse across membranes and directly influence secondary messenger systems without having to bind to membrane receptors first (Dhir & Kulkarni, 2011; Yun *et al*., 1997). An example relevant to MDD is the NO mediated increase in cyclic guanosine monophosphate (cGMP) (Dhir & Kulkarni, 2011). This is accomplished by the activation of soluble guanylate cyclase in the cytosol which stimulates the dephosphorilation of guanosine triphosphate (Dhir & Kulkarni, 2011). cGMP has been shown in various studies to increase depressive-like behaviour (Dhir & Kulkarni, 2011). It has been shown that NO is also involved in damage caused due to oxidative and nitrosative stress (Esplugues, 2002; Yun *et al*., 1997). It reacts with superoxide to form peroxynitrate, a potent reactive nitrogen species (RNS) (Beckman *et al*., 1990; Riddle *et al*., 2006). Damage caused by these molecules will be discussed in further detail in 2.4.6.

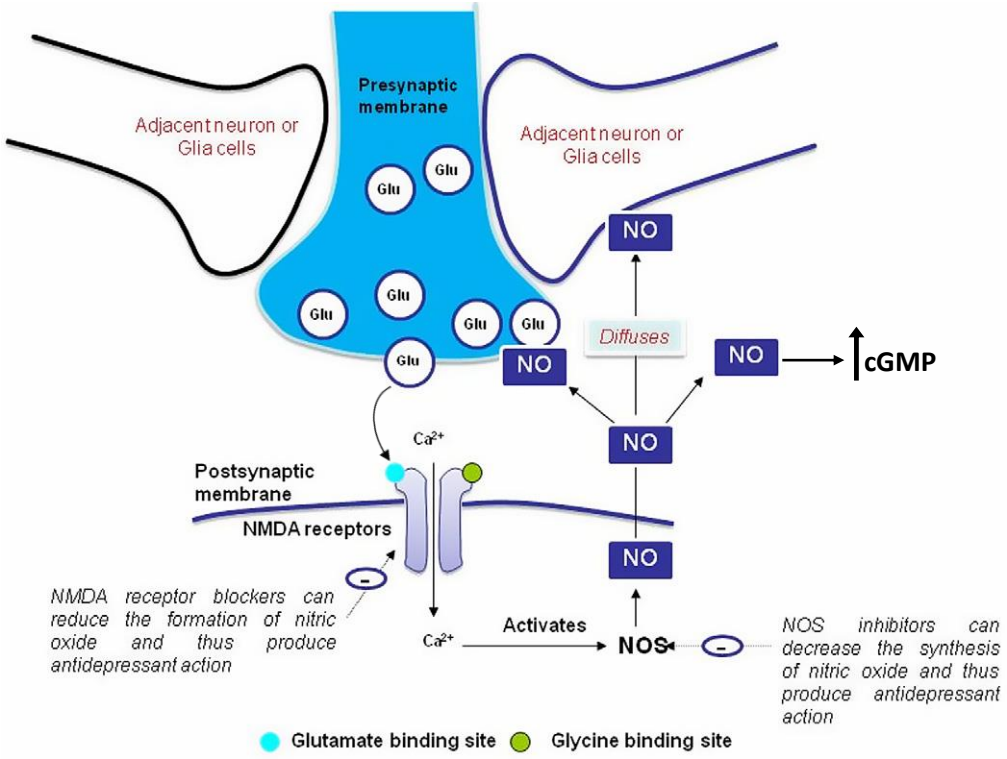

Figure 0-1: Synthesis of nitric oxide through the activation of NMDA receptors and calcium ion mobilization. Nitric oxide formed diffuses through the cell membranes and acts on the same neuron or adjacent neuron/glia cells and produced its action (adapted from Dhir & Kulkarni, 2011). Glu= glutamate, NOS= nitric oxide synthase, NO= nitric oxide, NMDA= N-methyl-D-aspartate, Ca^{2+}= calcium ions, cGMP= cyclic guanosine monophosphate.

Findings from various laboratories suggest a more complex role for NO in the development of MDD. Dhir and Kulkarni (2007) showed that the antidepressant effects of bupropion (a dopamine reuptake inhibitor is reversed by administration of sildenafil (a phosphodiestrase type 5 inhibitor (PDE5)). PDE5 inhibitors cause an increase in cGMP by preventing the

conversion of this substance to guanosine monophosphate (Dhir and Kulkarni, 2011). With this in mind the antidepressant effect of bupropion appears to be due to its ability to decrease cGMP. Sildenafil has been shown to decrease depressive-like behaviour in other studies however when co-administered with atropine (an antimuscarinic agent) (Brink *et al*., 2008; Liebenberg *et al*., 2010). Taken together these findings suggest that the role of NO-cGMP system in MDD and its interaction with other molecular systems remains poorly understood.

Glutamate is known to be neurotoxic in high concentrations and therefore dysfunction in the above described system may lead to neuronal cell death in a process referred to as excitotoxicity (Bloom, 2006). Excitotoxicity exhibits a variety of features including: calcium dependent enzyme activation, reactive oxygen species (ROS) and RNS generation, NO formation, and apoptosis (Yamamato, 2010). Increased glutamate concentrations have been associated with various neurological and neuropsychiatric disorders (including MDD) in various studies, which suggest that MDD may be a neurodegenerative disorder (Hashimoto *et al*., 2007; Lan *et al*., 2009; Maeng *et al*., 2007).

2.4.6 Neuro-immunological/ Neuro-inflammatory Hypothesis of Depression

The role of the immunological system and the inflammatory process in the aetiology of neuropsychiatric disease has until recently been underestimated. I will now present some of the accumulating evidence which shows that dysfunction within the immunological system may in fact provide an integrative framework for many of the aetiological factors discussed. A remarkable similarity between sickness-behaviour and depression associated behaviours exists (Capuron & Miller, 2011). Sickness behaviour encompasses a range of behavioural changes seen in human and animal subjects suffering from infections. Examples are anhedonia, fatigue, decreased locomotor activity, reduced appetite, altered sleep patterns and also enhanced pain sensitivity (Hart, 1988; Kent *et al*., 1992). It has been shown in various experimental settings in both human subjects and animal studies that cytokines play a profound role in mediating and modifying sickness behaviour (Dantzer *et al*., 1998). Pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumour necrosis factor-α (TNFα) or administration of substances which cause the release of pro-inflammatory cytokines such as bacterial lipopolysaccharide (LPS) have been shown in various studies to illicit sickness behaviour (Leonard & Maes, 2012; Plata-Salamán and Borkoski, 1993). Furthermore, central administration of IL-10 (an anti-inflammatory cytokine) or insulin-like growth factor I, a growth factor with anti-inflammatory cytokine-like actions in the brain, decreases the severity of signs of sickness behaviour caused by central injection of LPS (Dantzer *et al*., 1998; Bluthé *et al*., 1999). A further finding of interest is that reversal of LPS injection induced sickness

behaviour can be accomplished by chronic (but not acute) administration of antidepressants (Yirmiya, 1996; Castanon *et al*., 2001). It has also been shown in several studies that SERT activity is enhanced by pro-inflammatory cytokines (Leonard & Maes., 2012). IL-1β and TNFα was shown to cause an increase in SERT by means of the p-38 dependent pathway (Zhu *et al*., 2006). Tsao and colleagues (2008) showed enhanced SERT activity in neuronal cell lines is caused by interferon gamma.

ROS and RNS are associated with both inflammation and cell mediated immunity (Maes *et al*., 2012). Effects of ROS such as hydrogen peroxide production are enhanced by pro-inflammatory cytokines (Maes *et al*., 2012). Cell-mediated immunity processes have also been shown to increase RNS formation (Maes *et al*., 2012). Both ROS and RNS have been shown to react harmfully with various cellular molecules including proteins, fatty acids, and nucleic acids (DNA: both nuclear and mitochondrial and ribonucleic acid (RNA)) (Che *et al*., 2010; Leonard & Maes, 2012). In fact, recent studies have shown that RNA may be more vulnerable than DNA when exposed to oxidative stress in the hippocampi of patients with neuropsychiatric disease (Che.*et al*., 2010). Damage to these molecules may also result in further activation of the immune system targeted towards the damaged molecules and tissues. This may cause further damage to these tissues via an autoimmune response (Maes *et al*., 2011a). A recent study conducted in our laboratory showed that both acute and chronic exposure to ozone caused an attenuated efficacy of imipramine (tricyclic antidepressant) to produce a decrease in depressive-like behaviour in Sprague-Dawley rats (Mokoena *et al*., 2010). It was also shown that that acute and chronic exposure to ozone results in increased oxidative stress in the frontal cortices of male Sprague-Dawley rats in the form of elevated levels of superoxide and malondialdehyde (a marker for lipid peroxidation) (Mokoena *et al*., 2011). According to Ballinger and colleagues (2005) inhaled ozone forms ROS by reacting with fluids lining the mucosal surfaces of the lungs.

There is also a growing body of evidence of the role of anti-oxidants in patients with MDD as well as animal studies of depression (Leonard & Maes., 2012). Examples of key antioxidants are coenzyme Q10, glutathione, and various vitamins (vitamins C and E) (Leonard & Maes., 2012). Certain enzymes also have specific roles in neutralising ROS and RNS. Examples are superoxide dismutase and glutathione peroxidase which are antioxidant enzymes that respectively neutralize superoxide and peroxide (Leonard & Maes., 2012). Haptoglobin and albumin (acute phase proteins) may also be considered to be functional as antioxidants since they bind ROS and RNS (Leonard & Maes., 2012). Cell membrane phospholipid damage by ROS is prevented by tryptophan and tyrosine residues (Moosmann & Behl, 2000). Tryptophan (Leonard & Maes, 2012; Reiter *et al*., 1999) and its metabolite, the

neurotransmitter 5-HT (Leonard & Maes, 2012; Wrona & Dryhurst, 1991), serve as functional antioxidants. As the production of central reactive oxygen species increases, the lower the concentration of 5-HT in the brain will be.

The brain's high metabolic rate and limited antioxidant resources render it particularly susceptible to oxidative and nitrosative stress (Leonard & Maes, 2012). Various authors have reported on direct links between MDD and a diminished antioxidant system. Tsuboi and colleagues (2006) found reduced α-tocopherol (vitamin E) and β-carotene in patients with high job stress. In this study it was shown that the high job stress subjects displayed significantly greater depressive symptoms measured by the Centre for Epidemiological Studies Depression Scale (CES-D) than low job stress participants. In another study it was found that depressed patients displayed lower plasma coenzyme Q10 concentrations (Maes *et al*., 2009). The investigators also showed that lowered coenzyme Q10 concentrations are associated with treatment resistant depression. Kodydkova and colleagues (2009) showed both glutathione and glutathioneperoxidase were reduced in woman diagnosed with MDD (DMS-IV criteria were used) in comparison to the non-depressed, age-matched control group. These results have been duplicated in various animal models of depression (Maes *et al*., 2011a). It has also been shown that antidepressant medication has the ability to restore antioxidant levels. Significant examples include an ex vivo study conducted by Schmidt and colleagues (2008) which showed that long-term treatment (24 h) with antidepressants (desipramine, imipramine, maprotiline and mirtazapine) significantly enhanced messenger RNA levels of antioxidant enzymes in human monocytic U-937 cells. In a study where rats were exposed to chronic mild stress, antidepressants were found to increase glutathione peroxidase activity within the brain (Eren *et al*., 2007a; Eren *et al*., 2007b). In a study conducted in 2009 by Kumar and Kumar it was shown that sertraline (a SSRI), stimulated recovery of a decrease in glutathione levels caused by toxins. Zafir and colleagues (2009) showed that treatment for 21 days with Fluoxetine hydrochloride (20 mg/kg/day), imipramine hydrochloride (10 mg/kg/day) and venlafaxine (10 mg/kg/day) re-establishes functionality of the following brain antioxidant systems following disruption caused by restraint stress: superoxide dismutase, catalase, glutathione S-transferase, glutathione reductase and glutathione levels. It was also showed that malondialdehyde and protein carbonyl levels were restored to normal due to treatment with the above antidepressants (Zafir *et al*., 2009). Furthermore, it is interesting to note these findings occurred for all three medications despite all of them belonging to different antidepressant classes (Zafir *et al*., 2009).

Tryptophan and its derivatives are also implicated under this hypothesis (Dantzer *et al*, 2008). Enhanced metabolism of tryptophan in the kynurenine pathway may be an important

contributing factor in the aetiology of MDD and other neuropsychiatric conditions (Dantzer *et al*, 2008). Since tryptophan is the precursor for 5-HT increased degradation of this molecule in the kynurenine pathway may cause a decrease in 5-HT synthesis (Dantzer *et al*, 2008). Furthermore, enhanced metabolism of tryptophan by this pathway may result in increased production of potentially harmful metabolites (Dantzer *et al*, 2008). Tryptophan catobolites include both neurodegenerative and neuroprotective molecules (Myint *et al*., 2007). In depression there is less tryptophan available to the brain, which is ascribed to lowered plasma tryptophan concentrations (DeMeyer *et al*., 1981; Maes, 2011). In addition cell mediated immunity activation via interferon gamma causes the induction of indoleamine 2,3-dioxygenase (Werner-Felmayer *et al*., 1989); the enzyme responsible for the formation of kynurenine (Myint *et al*., 2007). Kynurenine is formed in the liver by tryptophan 2,3-dioxygenase (refer to figure 2-2 for explanation) and up to 60% of kynurenine in the brain originates in these peripheral tissues (Myint *et al*., 2007). Kynurenine has been shown to have neurodegenerative effects. It has also been associated with the development of depression and anxiety (Maes *et al*., 2011b).

There are two metabolic pathways, depicted in Figure 2-2, that kynurenine can enter. In the first kynurenine is metabolised to 3-hydroxykynurenine and quinolinic acid (Chiarugi *et al*., 2001). 3-Hydroxykynurenine has the ability to induce neuronal apoptosis (Okuda *et al*., 1998). Quinolinic acid is a NMDA receptor agonist and has the ability to induce excitotoxicity (Schwarcz *et al*., 1983), a condition associated with neurodegenerative changes (see 2.4.5). In the other pathway kynurenine is metabolised to kynurenic acid. This substance has the ability to antagonise NMDA receptors (Perkins & Stone, 1982) and can, in this way, counteract the excitotoxicity induced by quinolinic acid (Stone & Darlington, 2002). If the balance of quinolinic acid mediated excitoxicity and kynurenic acid mediated neuroprotection is disturbed in such a way that the effects of quinolinic acid dominate, it is thought that this could contribute to the pathophysiology of MDD (Dantzer *et al*., 2008). It should be apparent that an increased activity of indoleamine 2,3-dioxygenase along with the findings of increased SERT activity mentioned earlier due to changes orchestrated by the immunological system may result in reduced 5-HT levels. Furthermore, the antioxidant activity displayed by tryptophan and 5-HT may cause additional decreases in 5-HT levels. This again illustrates the complexity and inter-relatedness of these various hypotheses since these findings appear to lend some support to the monoamine hypothesis of depression (discussed in 2.4.2) but provide a causal mechanism for the decreased 5-HT levels.

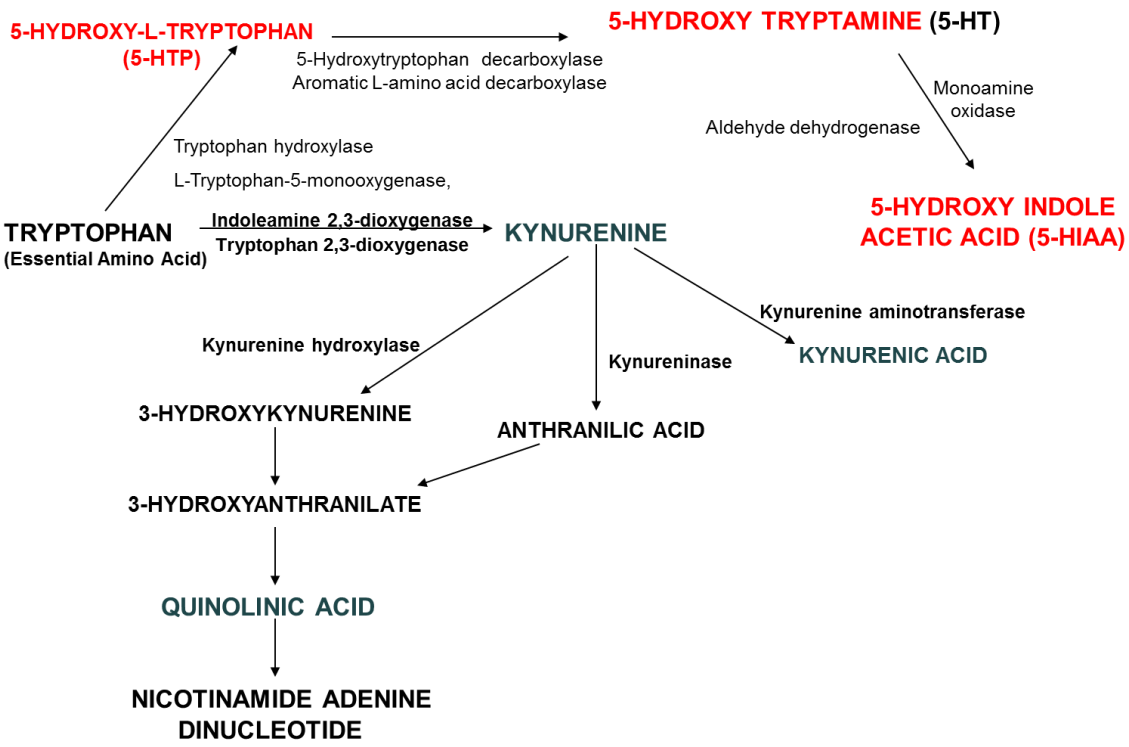

Figure 0-2: Metabolic pathways of tryptophan (Adapted from Maes *et al*., 2011b; Myint *et al*., 2007; Slopien *et al*., 2012).

2.5 Treatment Options for MDD

There are 6 main classes of antidepressants used clinically (Beldessarini, 2006). These include:

- Tricyclic antidepressants e.g. imipramine
- Monoamine oxidase inhibitors e.g. phenelzine
- Norepinephrine reuptake inhibitors e.g. reboxetine
- Serotonin reuptake inhibitors e.g. fluoxetine
- Serotonin-norepinephrine reuptake inhibitors e.g. venlafaxine
- Atypical antidepressants, e.g. agomelatine

The only FDA-approved antidepressants for children and adolescents suffering from MDD are fluoxetine and escitalopram, both SSRIs as mentioned earlier (Soutullo & Figueroa-Quintana, 2013). Several studies determined that SSRIs cause foetal developmental changes when used for MDD in pregnant mothers, but 5 year follow-up studies show no lasting negative effects in children exposed to these drugs *in utero* with regard to cognition, temperament, internalising and externalising behaviours (Mulder *et al*., 2011).

SSRIs act by inhibiting SERT proteins (see 2.7.2 for more information on synaptic clearance of monoamines). In this way the reuptake of serotonin into the presynaptic neuron is

prevented and this results in an increased synaptic concentration of 5-HT (Beldessarini, 2006). SSRIs have become the preferred treatment agent for MDD due to a better side effect profile than drug classes such as tricyclic antidepressants and monaomine oxidase inhibitors (Beldessarini, 2006). Various studies have shown that SSRIs have a greater therapeutic efficacy than tricyclic antidepressants in childhood and adolescent depression (Bridge *et al*., 2007; Mason *et al*., 2009). For these reasons fluoxetine is the recommended first-line drug for treatment of childhood and adolescent depression (Gentile, 2010; Nardi *et al*., 2013). Based on a number of studies, it is also recommended to avoid serotonin-norepinephrine reuptake inhibitors and older generation antidepressants in the treatment of MDD in juveniles (Nardi *et al*., 2013).

2.6 Animal Models of Depression

Various animal models of depression have been described, including (Overstreet & Wegener, 2013):

- 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 and resistant line rats

The Flinders sensitive line rat, a translational model of depression, was employed in this study. This well described and validated model of depression has been employed for 25 years and has been extensively used for a variety of studies designed to investigate various aspects of MDD, with more than 20 publications appearing between 2011 and 2012 (Overstreet & Wegener, 2013). Since this model has provided robust results in various studies of MDD, it was thought that it would prove to be an appropriate model for the study of the possible neurodevelopmental effects of fluoxetine.

The Flinders rat lines were initially developed to create a strain of rats resistant to the anticholinesterase agent diisopropyl fluorophosphate. This proved to be unsuccessful and the resulting rat line, denoted FSL rats, was shown to be more sensitive to diisopropyl fluorophosphate. The process of inbreeding also produced a second control line, denoted FRL rats, of which the sensitivity to diisopropyl fluorophosphate was comparable to that of Sprague-Dawley rats (Overstreet *et al*., 1979b). The enhanced sensitivity of FSL rats for cholinergic agonists is accompanied by a higher expression of muscarinic receptors (Daws and Overstreet, 1999; Overstreet & Russel, 1982). Subsequently it was noted that these rats

displayed greater depressive-like behaviour in tests, such as the FST. Following further validation in years to follow, this model has been demonstrated to be a useful rodent model of depression, which in several ways resembles human MDD. Janowski and colleagues (1994) showed that human depression is characterised by a cholinergic supersensitivity, thereby solidifying the similarities between the FSL model of depression and MDD sufferers.

But the validity of the FSL rat as translational model of MDD goes further. Three main types of validity need to be considered when assessing an animal model's reliability, namely face validity, construct validity, and predictive validity (Overstreet & Wegener, 2013). Face validity implies that the animal displays symptoms with correlates with those of the human condition (Overstreet & Wegener, 2013). Construct validity implies that there are biological correlates between the animal and human condition, such as corresponding abnormalities in neurological biomarkers or neurotransmission (Overstreet & Wegener, 2013). Predictive validity implies that the animal responds to the same drugs as the human condition and is resistant to drugs that the human condition also does not respond to (Overstreet & Wegener, 2013). In this sense, the model can predict human response to new drugs. Key facts of the FSL model will now be highlighted for each form of validity which shows that it is a reliable animal model of depression (Overstreet & Wegener, 2013):

Face validity:

1. Compared to FRL controls, FSL rats display more rapid eye movement (REM) sleep, a feature observed in depressed humans relative to unaffected humans (Benca *et al*., 1996; Shiromani *et al*., 1988).

2. Compared to FRL controls, FSL rats display increased passive behaviour following stress as displayed by greater immobility (reflective of increased depressive-like behaviour) during the FST (Overstreet & Wegener, 2013).

Construct validity:

1. Similar to observations in depressed humans, FSL rats show a decrease in 5-HT synthesis (Hasegawa *et al*., 2006).

2. Neuropeptide Y has been shown to be decreased in both humans with MDD (Wu *et al*., 2011) and FSL rats (Caberlotto *et al*., 1999; Jimenez-Vasquez *et al*., 2000).

3. FSL rats display cholinergic supersensitivity, similar to that found in human counterparts with MDD (Overstreet & Wegener, 2013).

4. Increased glutamate-NO signalling is evident in humans with MDD (Suzuki *et al*., 2001, other) as well as in FSL rats (Wegener *et al*., 2010).

Predictive validity:

1. All drugs effective in the treatment of MDD in humans cause a reduction in immobility, which is interpreted as a reduction of depressive-like behaviour, in the FST (Overstreet & Wegener, 2013).

2. With the exception of one drug, 3,4-methylenedioxymethamphetamine (Majumder *et al*., 2011), drugs need to be administered chronically (14 days) to cause a reduction in depressive-like behaviour (Overstreet & Wegener, 2013). This is also a major finding in humans suffering from depression (discussed in 2.4.2).

2.7 Monoaminergic Metabolism and Synaptic Clearance

In this study, we aimed to establish and implement the microdialysis technique for the measurement of monoamines in live-awake rats. It was hoped that we would be able to detect and measure the three major monoamines of central neurotransmission as well as the metabolites of these molecules. This would have allowed us to not only make meaningful conclusions about the synaptic release of the monoamines themselves but also their enzymatic turnover within the synaptic cleft as well as the clearance mechanisms involved in removing the monoamines from the synaptic cleft. In the context of the developmental framework of the study it would follow that early-life treatment might manifest as alterations of, amongst others, release and clearance mechanisms. It would therefore be prudent to briefly describe how these mechanisms function. It is also worth noting that the immunological system has been shown to significantly affect the expression and functioning of some of the reuptake transporters (as described in 2.4.6). Furthermore, preventing reuptake of neurotransmitters is the key mechanism by which the most frequently prescribed antidepressants for MDD work (discussed in 2.4.2).

2.7.1 Monoaminergic metabolism

A description of the synthesis of both DA and norepineprhine NE will now follow. NE and DA are synthesised from phenylalanine (Leonard, 2003). The first step of this process is the hydroxylation of phenylalanine with the aid of the enzyme phenylalanine hydroxylase (Leonard, 2003). The product of this reaction is tyrosine (Leonard, 2003). Tyrosine is metabolised by tyrosine hydroxylase to dopa, which in turn is converted to dopamine by means of an enzyme known as dopa decarboxylase (Leonard, 2003). NE is synthesised from DA by an enzyme known as dopamine-β-hydroxylase (Leonard, 2003).

NE and DA metabolism is depicted in Figure 2-3 below. According to Kvetnansky and colleagues (2009) the end products of NE and epinephrine metabolism in effector cells are 3-methoxy-4-hydroxyphenylglycol (MHPG) and vanillylmandelic acid (VMA). The end product of dopamine metabolism in effector cells is HVA.

DA may spontaneously degenerate into ROS and RNS (Miyazaki & Asanuma, 2008). Miyazaki and Asanuma (2008) suggested that auto-oxidation of DA to ROS and RNS is the major cause of DA induced cell death. Spontaneous ROS formation from DA produces superoxide which is converted enzymatically by superoxide dismutase to hydrogen peroxide (Miyazaki & Asanuma, 2008). Superoxide also reacts with nitric oxide to form the RNS peroxynitrite as described in 2.4.5. Furthermore, hydrogen peroxide may form hydroxyl radicals by reacting with metal species such as iron (Miyazaki & Asanuma, 2008). This radical is known to be the most cytotoxic radical (Miyazaki & Asanuma, 2008). Additionally, normal metabolism of DA by monoamine oxidase results in the formation of 3,4-dihydroxyphenylacetic acid and hydrogen peroxide (Miyazaki & Asanuma, 2008). These findings suggest that DA may play an important role in the processes associated with neuro-immunological/ neuro-inflammatory hypothesis of MDD.

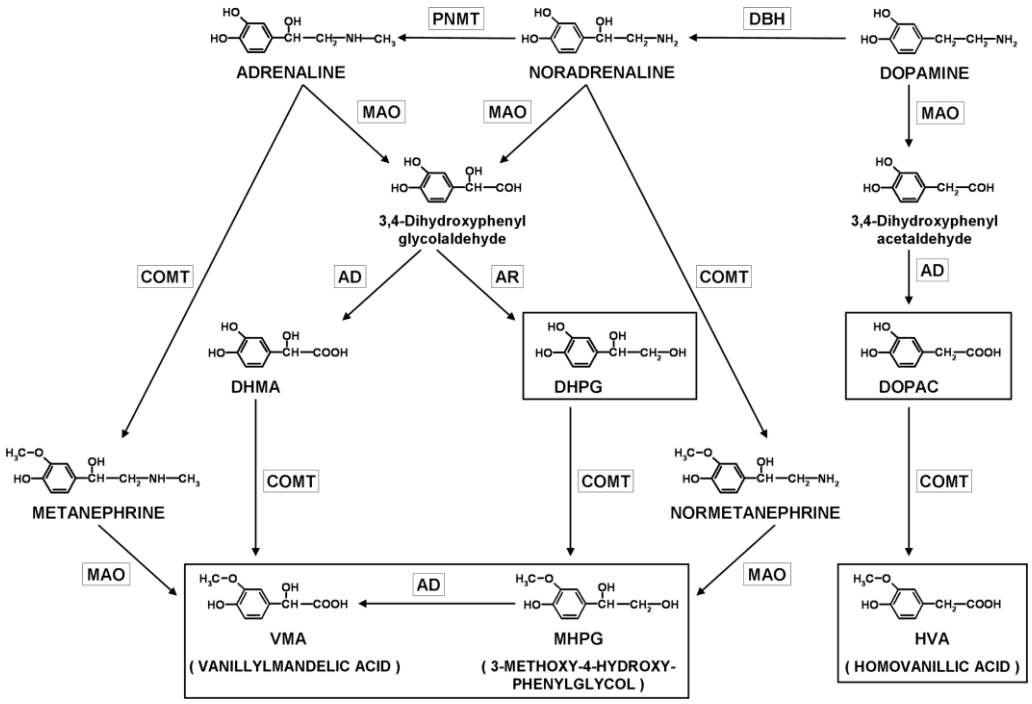

Figure 0-3: Norepinephrine and dopmaine metabolism (Kvetnansky et al., 2009). PMNT= phenylethanolamine N-methyltransferase; DBH= dopamine-β-hydroxylase; MAO= monoamine oxidase; COMT= catechol-O-metyltransferase; AD=aldehyde dehydrogenase; AR= aldehyde reductase; DHMA= 3,4-dihydroxymandelic acid; DHPG= 3,4-dihydroxyphenylglycol; DOPAC= 3,4-dihydoxyphenylacetic acid; VMA= vanillylmandelic acid; MHPG= 3-methoxy-4-hydroxyphenylglycol; HVA= homovanillic acid.

As can be seen in Figure 2-2 in 2.4.6 5-HT is synthesised from an essential amino acid named tryptophan. Tryptophan is initially converted to 5-hydroxy-l-tryptophan (5-HTP). This molecule is in turn metabolised to 5-HT. The metabolite of 5-HT is 5-HIAA (Maes *et al*., 2011b; Myint *et al*., 2007; Słopień *et al*., 2012). As described earlier in 2.4.6 tryptophan can be metabolised in in one of two pathways. In one pathway 5-HT and its metabolites will form and in the other (kynurenine pathway) potentially damaging metabolites might form. Furthermore, cell mediated immunity activation via interferon gamma causes the induction of indoleamine 2,3-dioxygenase the enzyme which may lead to decreased 5-HT (and therefore 5-HIAA) which is associated with the development of MDD formation and increased formation of kynurenine (and damaging metabolites).

2.7.2 Monoaminergic Clearance

2.7.2.1 Specific Monoaminergic Clearance

The concept of neurotransmitter uptake as a means of inactivating neurotransmitters, as conceived by Herting and Axelrod in 1961, has proved to be immensely important given how many antidepressants rely on this mechanism. In the initial publication the process of NE reuptake by sympathetic nerve terminals was described. The discovery of 5-HT and DA reuptake mechanisms soon followed (Iversen, 1971). In Figure 2-4 the process of reuptake from the synaptic cleft for NE, DA, and 5-HT is depicted. While SERT appears to be relatively selective for 5-HT, the norepinephrine transporter and dopamine transporter (DAT) have been shown to have the ability to transport both NE and DA (Torres *et al*., 2003).

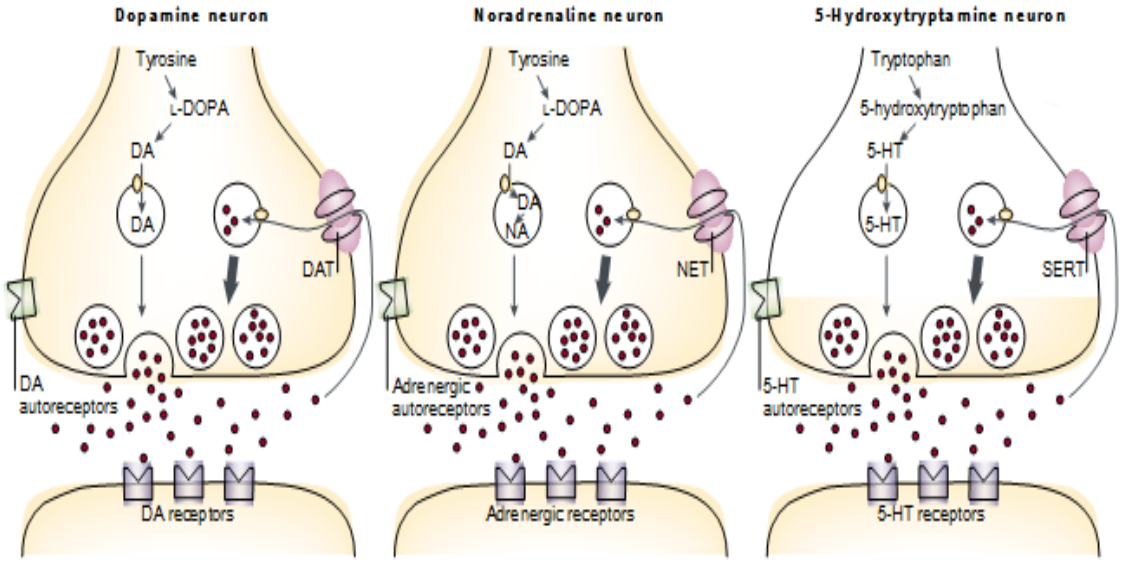

Figure 0-4: Dopamine, norepinephrine, and serotonin reuptake (Adapted from Torres et al., 2003). DAT= dopamine transporter, NET= norepinephrine transporter, SERT= serotonin transporter, DA= Dopamine, NA= Norepinephrine, 5-HT= Serotonin.

2.7.2.2 Nonspecific Monoaminergic Clearance

This type of reuptake is associated with a class of transport proteins referred to as organic cation transporters (OCTs) (Amphoux *et al*., 2006). These molecules exhibit bidirectional transport. Though they have low affinities (Amphoux *et al*., 2006) for the molecules that they transport, they are able to transport a high volume of these molecules (Busch *et al*, 1996; Grundemann *et al*, 1998). They are responsible for uptake of a variety of organic cations including NE, DA, and 5-HT (Amphoux *et al*., 2006; Busch *et al*., 1996; Gasser *et al*., 2006; Gründemann *et al*., 1998). These transporters were identified in the central nervous system (Amphoux *et al*., 2006; Wu *et al*., 1998) in a number of recent studies but also occur peripherally. Of the 3 subtypes identified up to now (OCT1, OCT2, and OCT3) all 3 are present in the central nervous system (CNS) (Gasser *et al*., 2006; Gasser *et al*., 2009; Taubert *et al*., 2007). Compared to the other two subtypes, OCT1 appears to have the lowest messenger RNA expression in the brain (Amphoux *et al*., 2006). It is only present in noteworthy amounts in brain areas where DA is abundant (Taubert *et al*., 2007) and also in the choroid plexus, leptomeninges and the ependymal layer of the third ventricle (Amphoux *et al*., 2006). OCT2 and OCT3 are distributed more extensively in the brain and are expressed in high numbers in the dorsomedial hypothalamus (Gasser *et al*., 2006; Gasser *et al*., 2009; Vialou *et al*., 2004). Baganz and colleagues (2008) demonstrated that in brains of mice with reduced SERT expression, OCT3 expression is upregulated. Furthermore, it was shown that administering an OCT3 antagonist (decynium-22) prevented removal of 5-HT from the synapse and had antidepressant-like effects in the tail-suspension test when compared to wild-type mice. It has also been suggested that OCT3 specifically may be involved in regulation of physiological stress responses due to the fact that it is sensitive to inhibition by systemic corticosterone (Gasser *et al*., 2006; Wu *et al*., 1998).

2.8 Monoaminergic Neurotransmitter Nuclei and Pathways

2.8.1 Noradrenergic Nuclei and Pathways

Figure 2-5 depicts the noradrenergic pathways of the rat brain. Seven noradrenergic nuclei (A1 to A7) have been described in rats by Dahlstrom and Fuxe half a century ago (Szabadi, 2013). These nuclei were also been described in humans by Bogerts in 1981 (Szabadi, 2013) and other primates (Felten and Sladek, 1983). According to Szabadi (2013), the noradrenergic nuclei are usually subdivided into three groups. The group of greatest importance in man (half of all the noradrenergic neurones are contained in it) is known as the rostral or pontine group (Szabadi, 2013). A6 is the sole nucleus in this group and

comprises the locus coeruleus (Szabadi, 2013). The A3 nucleus is absent in primates (Szabadi, 2013).

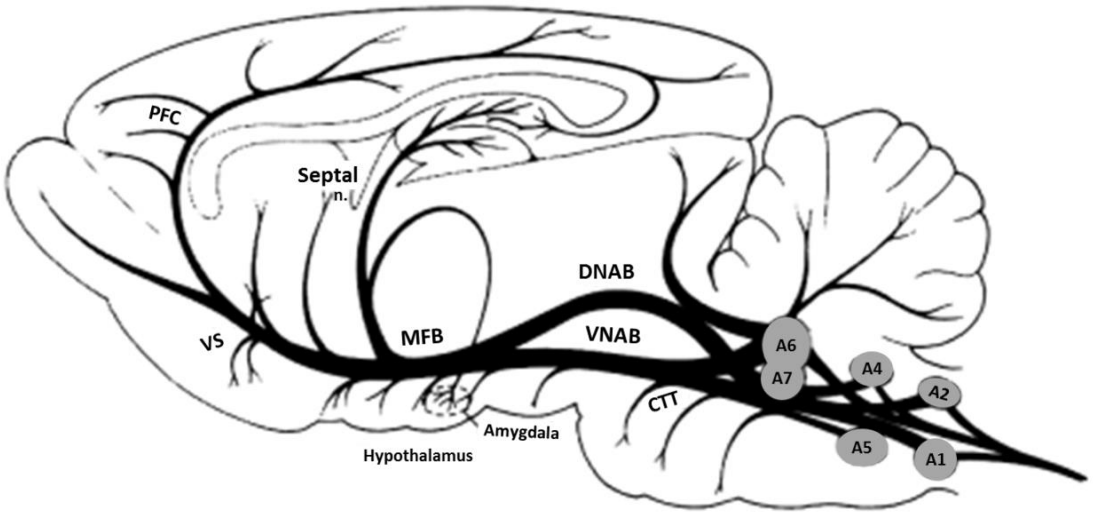

Figure 0-5: Noradrenergic pathways in the rat brain (Robbins and Everitt, 1993). DNAB= dorsal noradrenergic signalling bundle, CTT= central tegmental tract, MFB= medial forebrain bundle, PFC= prefrontal cortex, VNAB= ventral noradrenergic ascending bundle, VS= ventral striatum, n= nerves.

Figure 2-6 depicts the noradrenergic pathways of the human brain. According to Leonard (2003), the most important noradrenergic neurons implicated in psychological states originate within the locus coeruleus. From this structure located in the brainstem, fibres ascend to the following areas: thalamus, dorsal hypothalamus, hippocampus, and the cortex (Leonard, 2003). There are two main noradrenergic fibre bundles which originate in the locus coeruleus (Leonard, 2003). The ventral noradrenergic bundle fibres project to the hypothalamus and subcortical limbic regions, whereas the dorsal noradrenergic bundle fibres project to the cortex (Leonard, 2003). Both these structures appear to be involved in motivation and reward (Leonard, 2003). They have also been associated with REM sleep which, as discussed in 2.5, may be disturbed in both patients with MDD and the FSL genetic rat model of depression (Leonard, 2003).

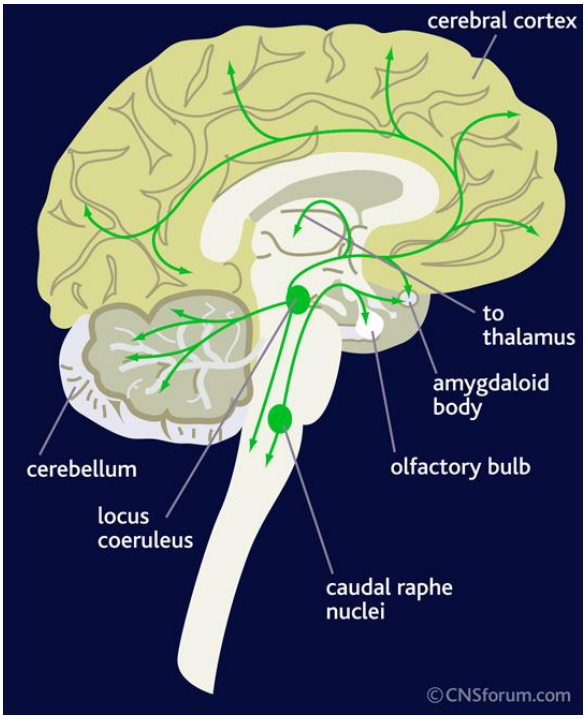

Figure 0-6: Noradrenergic pathways in the human brain (Lundbeck institute, 2014a).

2.8.2 Dopamine Nuclei and Pathways

Figure 2-7 depicts the dopaminergic pathways of the rat brain. The nuclei of cell bodies in the dopaminergic system in the mesencephalon of the rat brain are referred to as A8 (lateral tegmentum), A9 (substantia nigra) and A10 (ventral tegmentum) and corresponds well to primate structures (Schofield & Everitt., 1981). Dopamine fibre bundles A9 and A10 project to the PFC, striatum and nucleus accumbens (Miller *et al*., 2013).

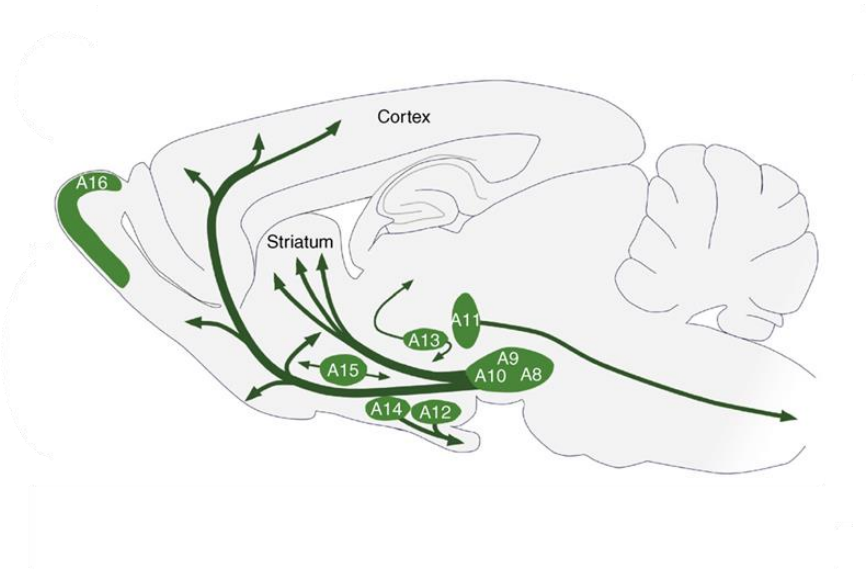

Figure 0-7: Dopaminergic pathways of the rat brain (Bjorklund & Dunnett, 2007).

Figure 2-8 depicts the dopaminergic pathways of the human brain.The mesolimbic pathway (commonly referred to as the “reward pathway”) originates in A10 and projects to the nucleus accumbens (Schultz, 2001). The nigrostriatal pathway comprises projections which originate in the substantia nigra and end in the striatum and is involved in motor control (Miller *et al*., 2013). Dysfunction in this pathway is associated with movement disorders such as Parkinsons disease and Huntington’s Chorea (Leonard, 2003). The third pathway is referred to as the mesocortical system (Miller *et al*., 2013). These dopaminergic fibres project from A10 to neostriatal areas such as the caudate and putamen (Miller *et al*., 2013; Leonard, 2003). Other fibres project to cortico-limbic structures such as the medial PFC, cingulate, and entorhinal areas (Leonard, 2003). Lastly, fibres in this pathway also project to structures which form part of the limbic system such as the septum, nucleus accumbens, amygdaloid and piriform complexes with various cognitive functions such as attention and memory associated with them (Leonard, 2003).

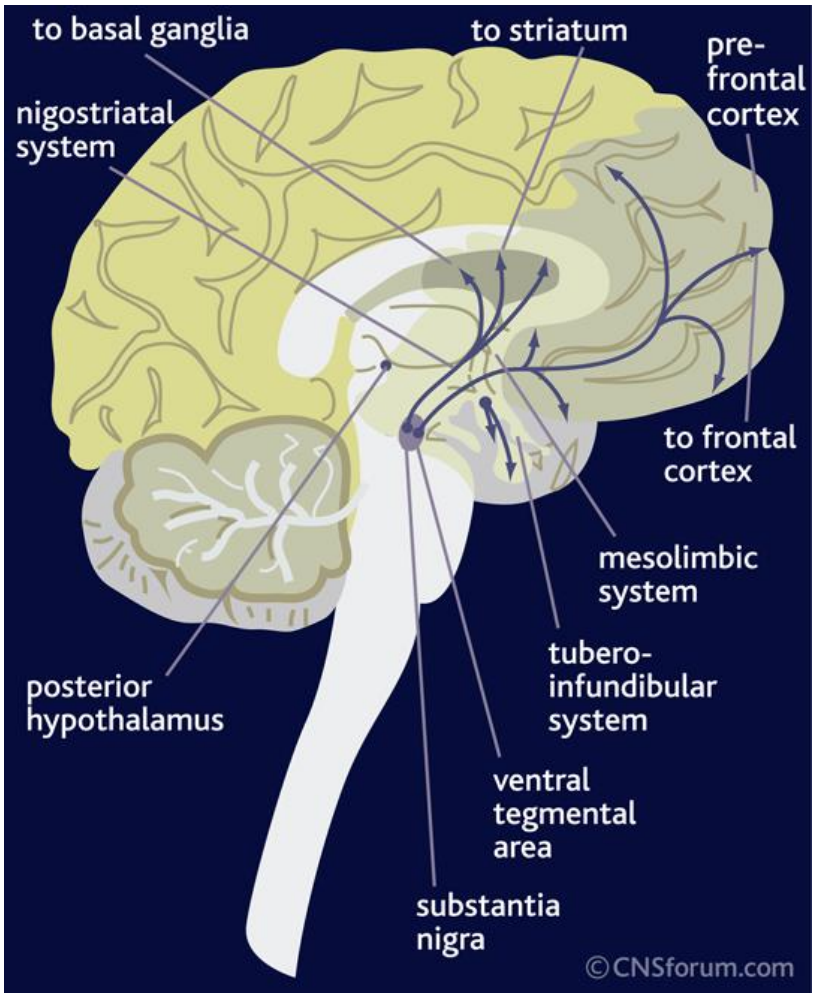

Figure 0-8: Dopaminergic pathways of the human brain (Lundbeck institute, 2014b).

2.8.3 Serotonin Nuclei and Pathways

Figure 2-9 depicts the serotonergic pathways of the rat brain. According to Jacobs and colleagues (1992), nine nuclei of the serotonergic system have been identified (B1 to B9). These nuclei are divided into 2 groups. The first group is the inferior brain stem group and the following nuclei are assigned to it: B1 and B4 (collectively referred to as nucleus raphe pallidus), B2 (nucleus raphe obscurus), B3 (nucleus raphe magnus), neurons in the ventrolateral medulla known as the lateral paragigantocellular nucleus, B1 and B3 (intermediate reticular nuclei in the area postrema). The second group, known as the superior brain stem group comprise the following nuclei: B5 and B8 (median raphe nucleus and its laterally displaced cells in nucleus pontis centralis oralis), B6 and B7 (the dorsal raphe nucleus), B8 (caudal linear nucleus), lateral B9 neurons situated dorsal to the medial lemniscus. According to Jacobs and colleagues (1992), rats and primates share all six of the serotonergic brainstem fibre bundles. The superior group of nuclei serve as the major origin for serotonergic fibres which project to the forebrain.

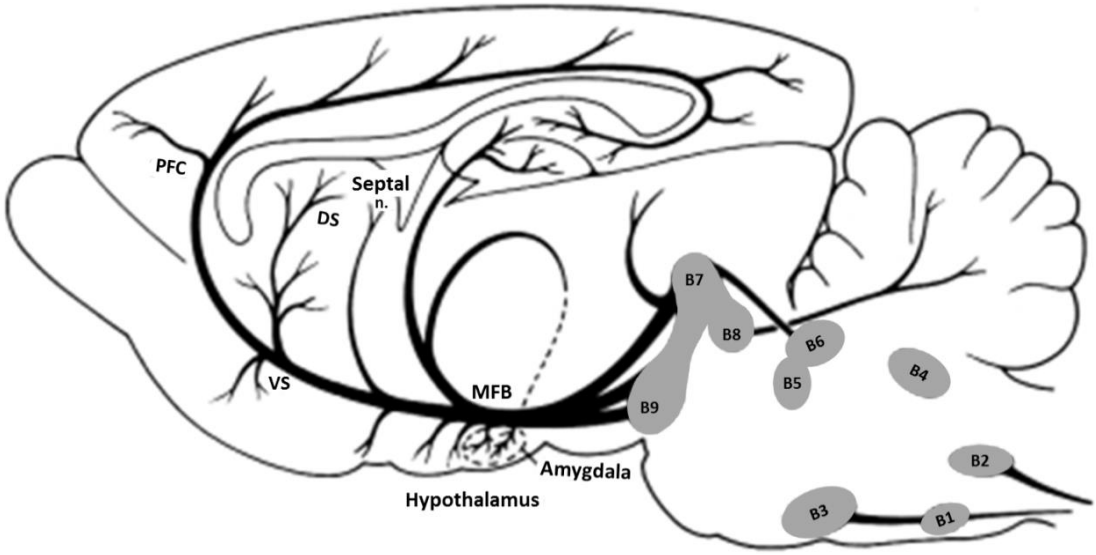

Figure 0-9: Serotonergic pathways of the rat brain (Robbins and Everitt, 1993). DS= dorsal striatum, MFB= medial forebrain bundle, PFC= prefrontal cortex, VS= ventral striatum, n= nerves.

Figure 2-10 depicts the serotonergic pathways of the human brain.According to Leonard (2003), fibres from the rostral portions of the median and dorsal raphe project to limbic areas such as the hippocampus and cortex.

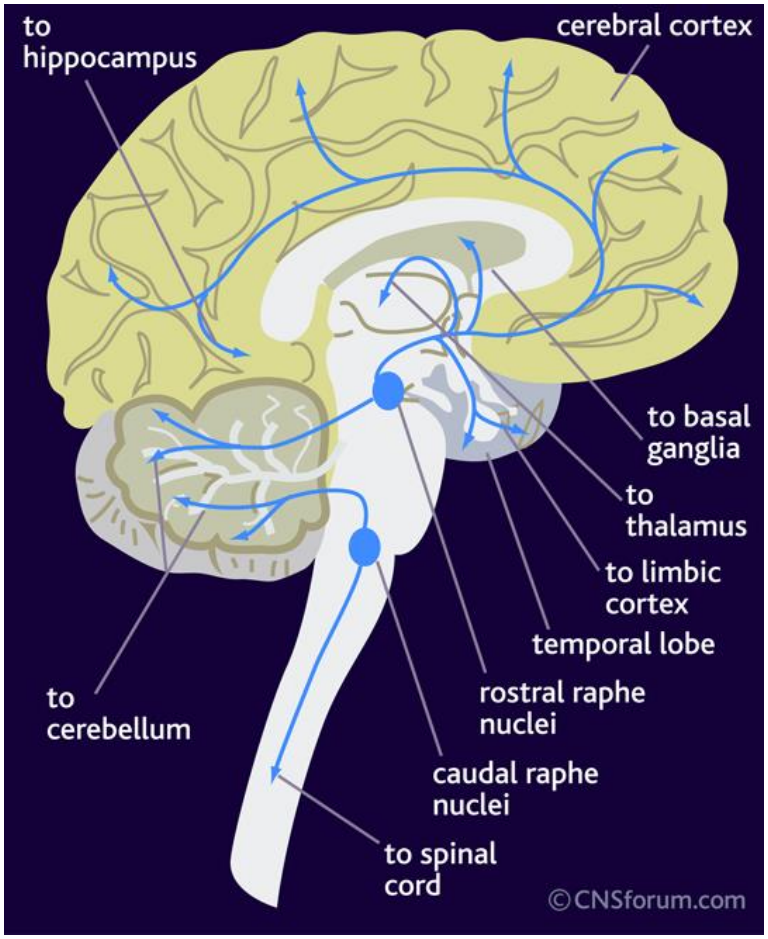

Figure 0-10: Serotonergic pathways of the human brain (Lundbeck institute, 2014c).

2.9 Stress and Monoamines

According to Kvetnansky and colleagues (2009), stress is any stimulus or process which disturbs homeostasis. Acute stressors consist of single or intermittent exposures to stress or continuous exposure to stress over a short period (Kvetnansky *et al*., 2009). Chronic stressors consist of continuous or multiple episodes of stress over an extended period of time (Kvetnansky *et al*., 2009). Stressors can also be further subdivided into 4 groups according to the nature of the stressor (Kvetnansky *et al*., 2009). The first group consists of physical environmental stressors such as temperature variations or extremes, solar radiation, intense noise and light intensity, and immobilisation, exposure to certain chemicals, and noxious stimuli (Kvetnansky *et al*., 2009). The second group comprise psychological stressors which are responsible for upsetting the emotional state and mood (Kvetnansky *et al*., 2009). The third group of stressors are social in nature (Kvetnansky *et*

al., 2009). This type of stressor is associated with compromised functioning in, or pressure from interactions of individuals within social groups (Kvetnansky *et al*., 2009). Examples of such stressors include unemployment and unexpected death of a loved one (Kvetnansky *et al*., 2009). The fourth and last group of stressors include those which induce changes within cardiovascular and metabolic homeostasis such as exercise, hypoglycaemia and excessive bleeding (Kvetnansky *et al*., 2009).

Rueter and Jacobs (1997) reviewed the evidence of the effect of stressors of various types on extracellular 5-HT concentrations. They reported that 5-HT concentrations increase by 30-100% following the introduction of acute stress in virtually all the brain areas studied. According to Kvetnansky and colleagues (2009), acute stress causes brief activation of the HPA axis (See 2.3.2). This is caused by alterations to catecholamine (CA) systems such as NE and DA neurons in the brain. After cessation of the stressor homeostasis is usually restored. However the nature and intensity of the stressor are factors which may determine whether normal CA functioning is restored. Chronic stress results in sustained functional alterations and increased activity of these neurons (Mamalaki *et al*., 1992; Rusnák *et al*., 2001; Watanabe *et al*., 1995). Yet, during stress sustained over long periods of time the synthesis of CAs may not meet the demand placed on the system resulting in reduced CA levels available for release at the synapse (Kvetnansky *et al*., 2009).

2.10 Neural Development

It has been well described that children and adolescents respond differently to antidepressant treatment than adults (Bylund & Reed., 2007). Whereas adults respond to SSRIs, TCAs and other classes of antidepressants, children and adolescent MDD patients tend to respond moslty to SSRIs (Bylund & Reed., 2007). Puig-Antich and colleagues (1987) found a similar response rate when placebo was compared to imipramine, a tricyclic antidepressant, in a randomised control trial of 16 teenagers. According to Burns and colleagues (1999), it has been demonstrated in numerous studies that tricyclic antidepressants show no benefit in children with MDD when compared to placebo. Hughes and colleagues (1990) found that 62% of the 12–18 year olds with MDD, diagnosed before puberty, continued to experience symptoms associated with MDD when undergoing treatment with imipramine. In a double-blind randomised control trial, imipramine showed no significant therapeutic effect relative to placebo, whereas paroxetine was effective and well tolerated when used for the treatment of adolescent MDD (Keller *et al*., 2010). It is hypothesised that this may be explained by the early development of the serotonergic system relative to other neurotransmitter systems (Bylund & Reed., 2007).

Great concern exists over the potentially profound neurodevelopmental changes which may be caused by the treatment of children and adolescents with SSRIs (Murrin *et al*., 2007). The rapid development and wide distribution throughout the central nervous system suggests that it may be involved in the maturation of many other cells in the brain (Whitaker-Azmitia, 2005). It has been postulated that the brain responds to the challenges of the environment by means of neurodevelopmental changes, which allows favourable responses to these challenges (Ansorge *et al*., 2008). It follows that changes in neurodevelopment induced by an increase in the synaptic concentration of 5-HT (e.g. SSRI-mediated) may potentially also affect the individual's ability to adapt (respond favourably) to environmental challenges. In this regard Ansorge and colleagues (2008) demonstrated that prenatal exposure to SERT inhibiting agents such as SSRIs, but not NET inhibitors, produce undesirable behavioural outcomes later in life.

Neurodevelopment has been extensively studied in various mammalian species, investigating various markers of development, and it has been found that the general order and patterns of neurodevelopment remain remarkably similar between most species, including humans and rodents (Murrin *et al*., 2007). However, the time frames for these changes differ significantly between species (Murrin *et al*., 2007). Due to the ease of housing and breeding of rodents, their relatively short life-cycle compared to humans and the considerable amount of existing data available on their neurodevelopment, they have become an attractive model to use in appropriate neurodevelopmental studies. As such, it takes 21 days from conception until birth, and another 35 days to puberty (Murrin *et al*., 2007). At around 60 days postnatal they are already in early adulthood (Murrin *et al*., 2007). We referred to all postnatal developmental ages relative to the day of birth (ND+). All prenatal (ND-) developmental ages are also described relative to the day of birth (e.g. ND-7). Accordingly the date of conception is referred to as ND-21. Some features of serotonergic, noradrenergic and dopaminergic neurodevelopment will now be highlighted. Important developmental milestones as shown in Figure 2-11 are discussed in the following paragraphs.

According to Murrin and colleagues (2007), the 8 mm rat embryo already has 5-HT-containing neurons and DA-containing neurons appear in the 9mm rat embryo, but NE-containing neurons only appear in the 11 mm rat embryo (Figure 2-11). As shown in Figure 2-11, by ND-04 serotonergic neurons have completed projections to adult-like pathways, having started this process on ND-07 (Wallace & Lauder, 1983). Noradrenergic neurons differentiate in the rat at ND-11 to ND-08 (Lauder and Bloom, 1974). By ND-02 the noradrenergic system has not appreciably developed much further, whereas the

serotonergic system, shown in Figure 2-11, resembles what is seen in the adult rat (Wallace & Lauder, 1983; Aitken & Tork, 1988). A pattern has emerged in the maturation of the serotonergic system beginning on ND+07, where there is an initial rise in serotonergic neurons to levels exceeding those seen in the adult rat (Figure 2-11) (Murrin *et al*., 2007). This rise in serotonergic neurons also corresponds to a 20% increase in 5-HT-labelled varicosities compared to levels found at birth as observed by Dinopoulos and colleagues (1997). It is suspected that this rise in 5-HT levels may significantly impact on further neurodevelopment. The 5-HT levels then decline gradually to adult-like levels around the third post-natal week (Andersen & Navalta, 2004). According to Murrin and colleagues (2007), the serotonergic dendrites have grown by the end of the first postnatal week into a form resembling that of the adult, but only by the end of the third postnatal week the adult serotonergic dendrite pattern has been established (Murrin *et al*., 2007) and the serotonergic innervation pattern of the cerebral cortex of the rat has fully developed (Figure 2-11) (Dinopoulos *et al*., 1997). The cortical development of noradrenergic neurons is initiated on ND+08 and continues throughout early postnatal maturation. There is also a marked difference between synaptogenesis between the two neurotransmitter systems with 75% of serotonergic synaptogenesis (in the raphe nucleus) and only 55% of noradrenergic synaptogenesis (in the locus coeruleus) completed by ND+15 (Figure 2-11) (Lauder and Bloom, 1974). The development of the serotonergic neurotransmitter system is completed by ND+21 whereas the noradrenergic neurotransmitter system continues to develop up to ND+35 (Murrin *et al*., 2007) as illustrated in the Figure 2-11.

In 1988, Kalsbeek and colleagues described the dopaminergic neuro-development in the pre-frontal cortex. On ND-17 DA-containing fibres arrive at the anlage of the lateral neocortex (Figure 2-11). By ND-15 DA-containing neurons appear at the subplate of the immature PFC (Figure 2-11) (Kalsbeek *et al*., 1988). Preceding birth dopaminergic fibres begin to move into the cortical plate as evidenced by the presence of dopaminergic fibres in the marginal zone of the lateral and the medial walls of each cerebral hemisphere shown in Figure 2-11 (Kalsbeek *et al*., 1988). By ND+2 the amount of dopaminergic fibres has increased considerably in the marginal zone. By ND+4 morphological changes of the DA fibres suggests the initiation of dopaminergic innervation (Figure 2-11) (Kalsbeek *et al*., 1988). At ND+6 the PFC can be divided into subareas based on the distribution of DA fibres (Figure 2-11). Dopaminergic fibre density increases until ND+60 as seen in Figure 2-11 (Kalsbeek *et al*., 1988). No further changes in DA fibre densities were observed between ND+60 and ND+90 (Kalsbeek *et al*., 1988). Both glutamate and gamma-aminobutyric acid activity are subject to modulation by DA in the PFC (O'Donnell, 2010). It follows that

developmental alterations within the dopaminergic system may give rise to beneficial or detrimental effects in the development of MDD later in life. Despite the success of MDD treatments targeting both the serotonergic and noradrenergic systems, developmental dopaminergic changes should not be ignored given its possible role in three of the major aetiological hypotheses of MDD (see 2.4.3, 2.4.5, and 2.4.6 for the monoamine, glutamate, and neuro-immunological/ neuro-inflammatory hypotheses of MDD).

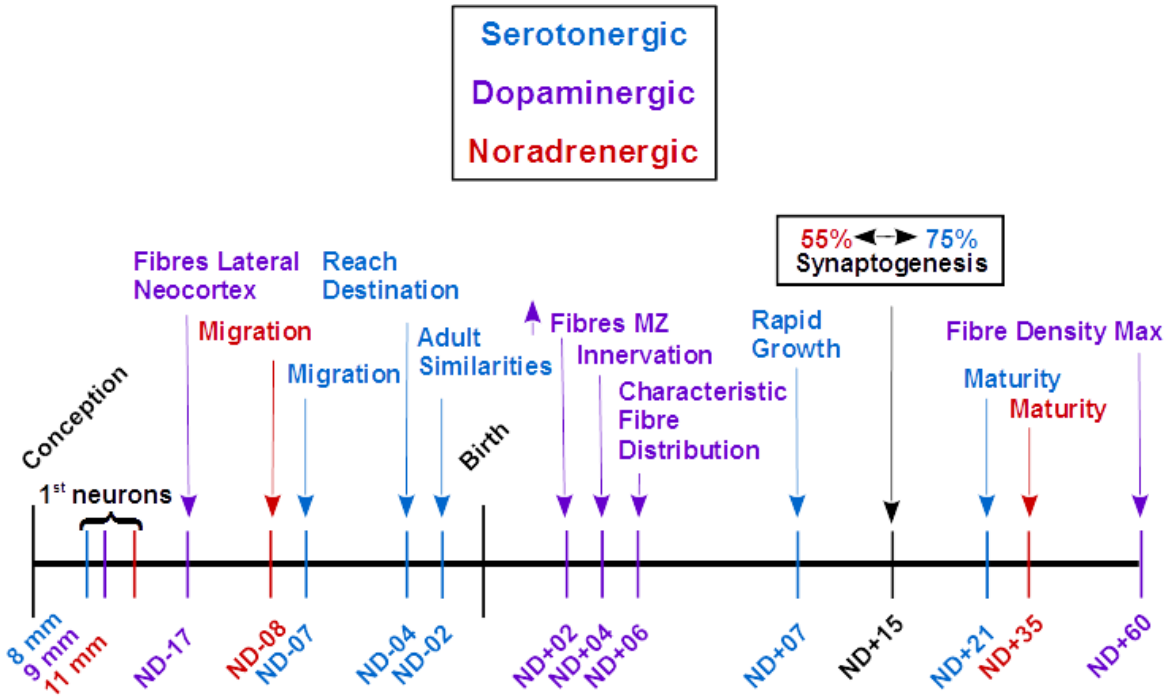

Figure 0-11: Developmental timelines of selected features of serotonergic, noradrenergic, and dopaminergic neurotransmitter systems (adapted from Steyn, 2011). ND = Natal Day, MZ = Marginal Zone, ↑ Increased.

It has been postulated that a fully developed, functional neurotransmitter system is necessary for a drug targeting that system to be demonstrably effective. Drugs that rely on the noradrenergic system (which attains maturity in early adulthood) for its antidepressant activity, such as the norepinephrine reuptake inhibitors, are less effective in childhood and puberty than those acting via the enhancement of serotonergic neurotransmission (which undergoes maturation before the onset of adolescence) (Bylund & Reed, 2007; Murrin *et al*., 2007).

This study follows on a previous study conducted by Steyn in 2011 in our laboratory which investigated “The effect of early-life exposure of stress-sensitive rats to the serotonin-norepinephrine reuptake inhibitor venlafaxine on behaviour in adulthood”. The findings of the study were as follows:

- Stress sensitive rats displayed decreased depressive-like behaviour in adulthood following pre- and/or postnatal venlafaxine treatment, when compared to stress-resistant control rats.
- Stress sensitive rats which received venlafaxine during pre- and/or postnatal life displayed, in adulthood, decreased depressive-like behaviour when compared to stress sensitive rats which received saline as vehicle control. This finding was not statistically significant however, possibly due to lack of statistical power.
- Stress resistant rats which received venlafaxine during pre- and/or postnatal life displayed, in adulthood, no significant alterations in depressive-like behaviour when compared to stress resistant rats which received saline as vehicle control.

It was therefore demonstrated that antidepressant treatment during adolescence may significantly reduce the depressive-like behaviour in stress sensitive rats. Since venlafaxine is a serotonin-norepinephrine reuptake inhibitor, and as such, is not approved for use in children and adolescents with MDD, the current study focussed on the potential neuroprotective and adverse effects of childhood and adolescent SSRI administration in young adulthood.

2.11 Synopsis

WHO has stated that major depressive disorder is the leading cause of disability world-wide, affecting a significant proportion of the global population: more than 350 million individuals including people of all ages, ethnicities, and socio-economic backgrounds (Marcus *et al*., 2012). There has also been an increase in the diagnosis and treatment of MDD and anxiety-related disorders in children and adolescents, and while possible reasons for this increase remain open to speculation (Zito *et al*., 2003), further elucidation is needed.

While adults suffering from MDD respond to drugs from various classes of antidepressants, children and adolescent MDD patients have only shown improvements due to treatment with SSRIs (Bylund & Reed, 2007) and only escitalopram and fluoxetine (SSRIs) have received FDA approval as antidepressants for children and adolescents suffering from MDD (Soutullo & Figueroa-Quintana, 2013). One possible explanation for this difference could be the early development of the serotonergic system relative to other neurotransmitter systems, implying that a neurotransmitter system needs to be fully developed and functional for medications which target that system to exert the desired effect (Bylund & Reed, 2007). Following this

hypothesis, it would be expected that drugs that rely on the noradrenergic system will therefore show decreased efficacy relative to drugs which target the serotonergic system (which completes development at an earlier age) (Bylund & Reed, 2007; Murrin *et al*., 2007).

There have been many studies conducted on SSRI-induced neurodevelopmental changes (Ansorge *et al*., 2008; Cabrera-Vera *et al.,* 1997; Freund *et al*., 2013; Hansen & Mikkelsen, 1998; Kepser & Homberg, 2014; Manhaes De Castro *et al.,* 2001; McNamara *et al*., 2008; Mulder *et al*., 2011; Rodriguez-Porcel *et al*., 2011; Soga *et al*., 2012). The hypothesized involvement of the serotonergic system in the maturation of other cells types and systems in the brain (Whitaker-Azmitia, 2005) has led to concerns being raised over potentially profound neurodevelopmental changes which may be caused by the treatment of children and adolescents with SSRIs (Murrin *et al*., 2007), the lack of studies on the role of the serotonergic system in neurodevelopment, and more specifically, the influence of serotonin levels on the development of other neurotransmitter systems has not yet been explored and this study aims to address this gap.

The role of environmental stress in the development of MDD (Belmaker & Agam, 2008) and the monoaminergic system's involvement in an individual's response to these stressors (Kvetnansky *et al*., 2009; Rueter & Jacobs, 1997) has been well described in the literature. Thus neurodevelopmental changes induced by SSRIs may be demonstrated by developmentally-exposed individuals' responses to environmental stressors.

In this study I investigated whether and how pre-pubertal fluoxetine administration, resulting in inhibition of SERT activity and a resultant increase in central 5-HT concentrations during pre-puberty influences the central neurodevelopment of other neurotransmitter systems. This was achieved by exploring the developmental effects of early-life fluoxetine administration on the noradrenergic and dopaminergic systems, the lasting effects within the serotonergic system itself and the effects of pre-pubertal fluoxetine administration on depressive-like behaviour, locomotor activity, and anxiety-like behaviour in FSL rats. Furthermore the monoaminergic stress response was measured using *in vivo* microdialysis in awake, freely moving FSL rats, a genetic rat model of depression. Corticosterone levels were also measured before and after exposure to a forced swim stress in order to ascertain whether the physiological stress response in rats exposed to microdialysis guide cannula placement surgery remained intact.

Chapter 3: Research article

The current dissertation is presented in the “article format”, as recognised 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 B. The references for this chapter will therefore be found at the end of the article manuscript. The references for the dissertation as a whole will 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, Discussion, Conclusions, Acknowledgements, References, Legends to Tables, Tables, Legends to Figures and Figures. However, for the benefit of 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.

N.J. Badenhorst 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 and L. Brand 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

The long-term effects of pre-pubertal fluoxetine on stress-related behaviour and acute monoaminergic stress response in stress sensitive rats

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Highlights

- Antidepressant use is increasing in children and adolescents.
- Potential developmental changes are a growing concern in this age group.
- Long-term changes after pre-pubertal fluoxetine treatment were assessed in rats.
- Fluoxetine treatment altered stress-induced prefronto-cortical monoamine release.
- Fluoxetine treatment significantly reduced locomotor activity.

Abstract

Fluoxetine and escitalopram are the only antidepressants approved by the Food and Drug Administration of the United States of America (FDA) for treatment of major depression in children and adolescents. Both drugs are selective serotonin reuptake inhibitors (SSRIs). In recent years there has been a growing concern over the long-term developmental effects of early-life exposure to SSRIs. In the current study we examined the developmental effects of pre-pubertal fluoxetine (10 mg/kg/day) administration in Flinders Sensitive Line (FSL)[1] rats on monoaminergic stress responses and behaviour. We found significant differences in monoamine concentrations between fluoxetine and saline (vehicle) control groups in response to an acute swim stress. The noradrenergic stress response appeared to be significantly supressed in fluoxetine treated FSL rats. The same trend, though not statistically significant, was visible for the serotonergic stress response. Prepubertal fluoxetine exposure increased baseline dopaminergic release significantly but no dopaminergic stress response was evident after exposure to an acute forced swim stress. A significant decrease in locomotor activity was observed in fluoxetine treated rats. This study therefore identified the dependence of the development of noradrenergic and dopaminergic systems on the serotonergic system. Furthermore, targets for future research to characterise the interactions between these neurotransmitter systems during development were identified, specifically serotonin receptor subtypes and monoamine transporter proteins.

Keywords

Neurodevelopment, Pre-puberty, Fluoxetine, Stress, Monoamines

[1] FSL: Flinders Sensitive Line
[2] FST: Forced Swim Test
[3] OFT: Open Field Test
[4] CA: Catecholamine

3.1 Introduction

Major depressive disorder (MDD) is a mood disorder affecting a significant proportion of the global population, in fact, according to the World Health Organisation it is the leading cause of disability world-wide [1]. More than 350 million individuals suffer from MDD [1]. The epidemiological data on MDD in the young reflect a similar MDD prevalence in adults and adolescents [2, 3, 4, 5]. In pre-puberty the prevalence of MDD is estimated to be 0-2% [6] and during mid to late adolescence the prevalence increases to 4-5% [7]. Some concerns were raised based on several reports suggesting that MDD is becoming a greater problem in juveniles. For example, more than a decade ago Zito and colleagues (2003) reported a rising trend in the number of antidepressant prescriptions for children and adolescents [8]. An increased suicide rate has also been observed in adolescents of the United States of America over a period of 40 years between 1950 and 1990 [3]. However, Costello and colleagues (2006) concluded in their meta-analysis of the available epidemiological data of MDD in the young that there is no evidence of a MDD epidemic within this population group and that the apparent increase of MDD in these age groups is more likely the result of an increasing awareness of a disorder which has, up to now, been underdiagnosed in children [3]. These findings were supported by a recent meta-analysis which found no evidence of a rise in prevalence of MDD and anxiety-disorders across all ages [9]. The authors site the growth of the global population as the reason for this perceived epidemic [9]. They also list an increased public awareness of these conditions and the use of the terms associated with MDD and anxiety disorders in non-clinical settings as major contributors to this impression [9]. The use of symptom checklists is also proposed as a possible cause since, according to the authors, they measure psychological distress and not clinical depression and anxiety disorders [9]. These conservative projections, however, do not disregard that juvenile depression is currently a significant and serious disorder across the world. In addition, it should be kept in mind that as the global population grows so does the amount of individuals affected by the disease.

Only fluoxetine and escitalopram, both SSRIs, has been approved by the FDA for the treatment of MDD in children and adolescents [10]. Several studies have shown that SSRIs cause foetal developmental changes when used for MDD in pregnant females [11, 12, 13]. However, 5 year follow-up studies suggested that in utero exposure does not have lasting negative effects on cognition, temperament, internalising, and externalising behaviours [14]. Prenatal exposure to serotonin (5-HT) transporter protein (SERT) inhibiting agents such as SSRIs, but not norepinephrine (NE) transporter (NET) inhibitors, were shown to produce undesirable behavioural outcomes later in life in a study conducted in mice [15]. A variety of

undesirable developmental outcomes have been described in previous studies [16] examining SSRI administration in late postnatal life in both mice and rats. These findings include alterations in reproductive behaviour [17], anxiety-like behaviour [17], social behaviour [18], and depressive-like behaviour [19]. Some studies have even shown neurochemical findings which resemble those associated with autism in humans [18, 20]. SSRIs like fluoxetine and escitalopram increase synaptic concentrations of serotonin by preventing its re-uptake via SERT [21]. Clinical studies have shown that SSRIs also inhibit the reuptake of norepinephrine and dopamine (DA); antagonize 5-HT_{2C}, muscarinic and sigma 1 receptors; and inhibit the synthesis of nitric oxide and various cytochrome P450 enzymes [22]. SSRIs prescribed to children and adolescents with MDD may therefore have profound consequences in terms of neurodevelopment. In a recent rat study it was demonstrated that prenatal exposure of rat pups to fluoxetine in stressed dams prevents increased depressive-like behaviour later in life as seen in untreated controls [23]. This study also presented evidence for improved hippocampal neurogenesis and cell proliferation in prenatally exposed rats compared to unexposed controls [23]. This opens up the possibility of improving at-risk individuals' outcomes with regard to depression later in life by appropriately using antidepressants when necessary in a preventive fashion early in life.

Stress has been shown to cause alterations in various systems implicated in MDD including monoaminergic neurotransmission, inflammation (due to the effects of the hypothalamic-pituitary-adrenal axis on immune system functioning) as well as neurogenesis and neuroplasticity [24, 25, 26]. Increases in serotonergic, noradrenergic and dopaminergic functioning has been described in previous animal studies as well [27, 28].

The current study investigated the possible developmental effects of fluoxetine (one of two antidepressants approved by the FDA for use in children with MDD) in a genetic rat model of depression, the FSL rat. This well described animal model of depression has been used extensively for over 25 years [30]. In this period it has shown reproducible face, construct and predicitive validity [30]. Since this model has provided robust results in various studies of MDD, it was thought that it would prove to be an appropriate model for the study of the possible neurodevelopmental effects of fluoxetine. Firstly, changes to neurobiological stress mechanisms which may be produced via early-life administration of fluoxetine were examined. Here we made use of microdialysis to investigate monoaminergic release and reuptake as well as enzymatic turnover in awake, freely moving rats before, during and after exposure to an acute stressor. We also explored the depressive-like behaviour, locomotor activity and anxiety-like behaviour in adult FSL rats injected with fluoxetine during pre-puberty.

Given the wealth of information available on SSRI-induced neurodevelopmental changes and the serotonergic system's early development relative to other monoaminergic systems it is surprising that studies on the influence of increased serotonin levels on other neurotransmitter systems have not been conducted. To our knowledge this is the first study which has examined the developmental effects of SSRI mediated increase in central serotonin concentrations on the neurodevelopment of other neurotransmitter systems.

3.2 Materials and methods

3.2.1 Subjects

A total of 56 male FSL rats were used in the study. All experiments were approved by the Ethics Committee of North-West University (ethical approval numbers: NWU-00045-10-5S and NWU-0028-08-A5) The rats were pair–housed in the North-West University vivarium with the environmental temperature maintained at 22 ± 1°C and humidity at 50%. A 12:12-h light/dark cycle (lights on at 06:00 and off at 18:00) was followed and food and water were available *ad libitum*. On postnatal day (ND+) 57 the rats used for the *in vivo* microdialysis and corticosterone assays were moved to the microdialysis lab maintaining the light/dark cycle and temperature, whereas rats for behavioural analyses were housed in the vivarium until testing on ND+60. The study layout is depicted in figure 3-1.

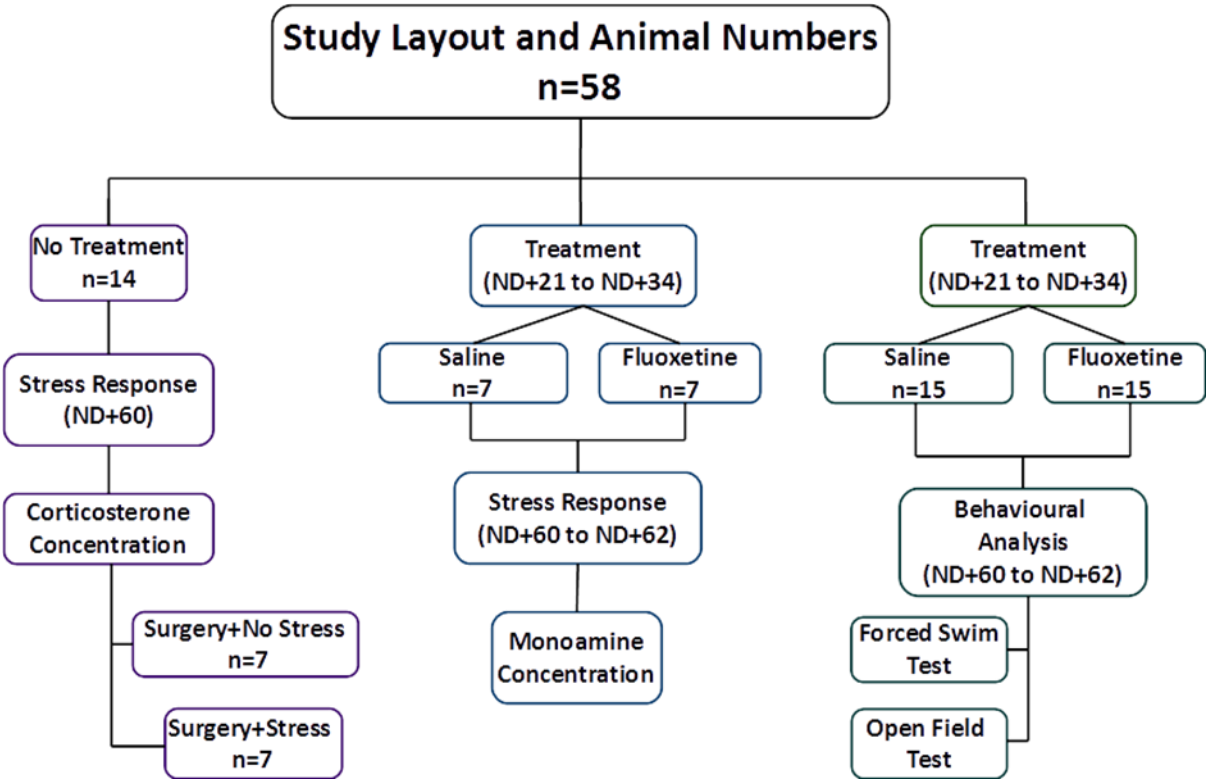

Figure 3-1: Study Layout

The first group of rats were used to determine baseline and post-stress corticosterone concentrations (see 3.2.4.2). The second group of rats were divided into two treatment groups. One received fluoxetine, the other received saline as vehicle control (see 3.2.2). The monoamine concentrations were determined in this group before, during and after a forced swim stressor (see 3.2.4.1). The third group, like the second group, was divided into two treatment groups and used to measure behavioural differences between treatment groups.

3.2.2 Drugs

Animals were treated with saline (vehicle) or fluoxetine (a kind gift from Aspen, Port Elizabeth, South Africa) subcutaneously (s.c.) once daily from ND+21 to ND+34. Fluoxetine HCl was administered at a dose of 10 mg/kg/day [29, 31], similar to previous studies in our laboratory and elsewhere where the drug was used for its antidepressant-like effect [29, 32, 33, 34, 35]. Rats were treated from ND+21 to ND+34, since this period represents a developmental phase during which the noradrenergic [36] and dopamenrgic [37] neurotransmitter systems are still developing, whilst serotonergic neurodevelopment had matured [36]. After treatment rats were randomly allocated for use in either microdialysis or behavioural experiments. For all microdialysis guide cannula placement surgeries (in the prefrontal cortex) anaesthesia was induced by intraperitoneal injection (0.1 ml/100g) of a mixture of xylazine (60mg/kg) and ketamine (10mg/kg) [38]. 5 ml of a 100% halothane solution (Halothane-M & B; Safeline Pharmaceuticals) was used to render animals motionless by placing them in an airtight enclosure to facilitate microdialysis probe placement on the third day after the surgery [39].

3.2.3 Behavioural analyses

30 male FSL rats were divided into two treatment groups. The first group received saline injections as saline control, the second group received fluoxetine injections. Treatment occurred from ND+21 to ND+34. All behaviour experiments were video-taped and scored by a researcher blinded to the treatment groups.

3.2.3.1 Forced swim test

The forced swim test (FST), first described by Porsolt in 1977, has been used successfully to monitor depressive-like behaviour in rodents for many years [40, 41]. It has since been modified by Lucki and colleagues (1997) in order to distinguish between behaviour mediated by the noradrenergic and serotonergic systems [42]. Immobility is defined as an absence of active movements other than those necessary to keep the animal's head above water. Secondly, swimming movements are defined as any horizontal movement, including those which involve crossing into another quadrant. Lastly, climbing behaviour is defined as any

upward-directed movement along the walls of the cylinder away from the water level [41]. The test was conducted in clear perspex cylinders (20 cm diameter and 40 cm high) [43]. On the day of the test the cylinders were filled to a depth of 30 cm with 25 ˚C water and the test was conducted under a light intensity of 200 lux [33, 41]. The typical conditioning swim 24 hours before the scoring session is not applicable to FSL rats [34]. The rats were placed in the water for 7 minutes. When scoring, the first and last minutes were ignored which means scoring was only done for a 5 minute period [33].The movements of the rats were scored according to the criteria laid out by Cryan and colleagues in 2002 [41].

3.2.3.2 Locomotor activity

Fatigue and anhedonia are known symptoms of MDD and lead to decreased general activity in the affected individual. In order to test for this phenomenon in rat models of depression locomotor activity is assessed as part of the open field test (OFT) [44]. The apparatus used for the test consisted of a square opaque Perspex container. The floor of the box served as a 1 m^2 test arena and was subdivided into sixteen 25 x 25 cm smaller squares [33]. In this test the total number of lines crossed within the 5 min testing session was used as an indication of general locomotor activity.

3.2.4 Stress response

3.2.4.1 Forced swim acute stressor

Kirby and colleagues (1997) showed that forced swimming was more effective as an acute stressor than tail-pinch, exposure to cold, immobilisation, or forced locomotion on a suspended rotating rod [45]. In this study we employed a 10 min forced swim as an acute stressor as employed in previous studies [46, 47]. The animals were gently dried after each swim (FST and acute swim stressor) with disposable paper tissue. Clear Perspex cylinders (20 cm diameter and 40 cm high) were used [43] filled to a depth of 30 cm with water at 25 ˚C ± 4 ˚C and under ± 200 lux white light [41]. Plasma corticosterone and prefrontal cortical monoamine levels were measured before, during (monoamine measurements only) and after this acute stressor.

3.2.4.2 Measurement of plasma corticosterone levels

Guide cannulas were placed in 14 Male FSL rats as described in 3.2.5, where after they were divided into two groups. One group was exposed to an acute swim stress, whereas the other group served as unstressed controls. Thereafter rats were decapitated and plasma corticosterone concentrations measured from trunk blood samples collected in 10 ml heparinised blood tubes. These samples were prepared and analysed (via HPLC) according to the method described by Viljoen and colleagues [48]. 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).

3.2.4.3 Measurement of monoamine levels in the prefrontal cortex

12 Male FSL rats received fluoxetine and saline treatments as described above, They were housed normally until ND+57 and moved to the microdialysis laboratory (described in 3.2.1). Thereafter rats were subjected to the acute swim stress (described in 3.2.4.1) and microdialysis (described in 3.2.5), for measurement of monoamine levels in the prefrontal cortex.

3.2.5 Microdialysis

After the pre-pubertal drug treatment, rats for microdialysis experiments underwent surgery for guide cannula placement in the prefrontal cortex. Anaesthesia was implemented as described in 3.2.2. Body temperature was maintained at 37 ˚C with a manually controlled surgical heating pad. A Kopf® stereotaxic frame was used to place the guide cannula 4.2 mm anterior-posterior (AP), 2.4 mm lateral (L), 2.4mm ventral (V) relative to bregma in the prefrontal cortex [48]. Three jeweller screws, 0.9 mm x 1.92mm, were placed shallowly into the bone to provide extra support and anchorage for the cannula [39, 50]. An intraperitoneal diclofenac injection, dose 2 mg/kg, was given after completion of the surgery for pain control [39]. The rat was then transferred to the microdialysis home cage and left to recover for 3 days prior to implantation of the microdialysis probe and commencement of the microdialysis procedure [39]. The subjects were monitored for signs of pain and infection during the recovery phase and immediately euthanised if any were noted [39].

On the day before the exposure to the forced swim stress (18:00) the rat was placed in an airtight cage with a halothane soaked cotton swab [39]. As soon as the rat became immobile it was removed and the probe was placed into the guide cannula before the subject recovered full consciousness [38]. The rat was then placed into the microdialysis enclosure and perfusion was initiated at 1.0 µl/min to allow equilibrium to be achieved prior to the microdialysis sampling procedure at 08:00 the next day [50]. The probe was placed on the evening prior to the sampling procedure [50] to reduce a halothane mediated increase or decrease in sample monoamine concentrations [51]. This decision was made based on a study conducted in our laboratory by Harvey and colleagues (2006) [51]. They showed that halothane causes an increase in frontal cortex NE concentrations and a decrease in frontal

cortex DA concentrations in whole brain tissue. 5-HT concentrations were not affected by halothane exposure [51]. Perfusion with artificial cerebrospinal fluid (aCSF) was initiated immediately after probe placement at a flow rate of 1.0 µl/min [39].

At 08:00 the next morning sampling commenced. Samples were collected over a time frame of 7 hours and 20 minutes. Samples were collected in 20 minute intervals yielding a sample volume of 20µl per sample. The first 3 samples were used to determine baseline levels of the monoamines. At the start of the fourth sampling interval the rat was placed in a FST cylinder filled with water (acute swim stress) for 10 minutes [46, 47]. The rat was removed from the FST cylinder and gently dried with tissue paper before being returned to the microdialysis enclosure. A further 18 samples were then collected over a period of 6 hours. The first sample collected in each hour after the swim stress was analysed, yielding 6 samples to investigate changes in monoamine concentrations for an extended time after the forced swim stress.

Isotonic aCSF, kept at room temperature, was used as perfusion fluid in this study [39]. Each sample was removed from the microfraction collector and immediately placed in an amber vial and placed in the auto-injector of the HPLC for analysis. Relative recovery was not determined in this qualitative study since the aim was to compare the results obtained in FSL rats treated with either fluoxetine or saline [39]. The aCSF was prepared by adding the following salts to ultra-pure water: NaCl (145 mM), KCl (3.0 mM), $CaCl_2$ (1.2 mM), and $MgCl_2$ (1.0 mM) [50]. The apparatus used included microdialysis probes (acquired from TSE systems) with 4 mm membrane working length and 6 kDa cut off. The syringe pump used was a MAB 40 Dual Channel Microdialysis Pump (by Microbiotech/Sweden). The refrigerated microfraction collector was an 820 Microsampler (by Univentor). Guide cannula placement surgeries were done with the aid of a Kopf® stereotaxic frame.

The technique described by Bert and colleagues (2004) was used to verify the probe position [52]. Computer software is used to superimpose a digital photograph of a coronal section of the harvested brain (of the experimental animal) onto a digital image of an appropriate stereotaxic atlas representation of a coronal section of the animal brain [52]. The track of the dialysis probe is visible and is used to verify the correct position of the probe against the atlas [52]. This technique was modified by combining it with a conventional staining procedure in order to enhance the ease and accuracy with which the investigator could determine the probe position

Immediately after the sampling procedure the rat was disconnected from the sampling apparatus and placed in the airtight halothane enclosure until immobile. Cresyl violet acetate

obtained from Sigma-Aldrich (CAS no: 10510-54-0, molecular formula: $C_{18}H_{15}N_3O_3$) was used. It was prepared by adding 0.1g of the cresyl violet acetate to 100 ml distilled water. Just prior to use 10 drops of glacial acetic acid was added and the solution was filtered [53]. The 0.1% Cresyl violet solution was injected via the inlet of the microdialysis probe, the rat was then placed back in the halothane enclosure for 7 minutes (recommended times 5-10 minutes) to allow the cresyl violet to stain the area of the brain adjacent the tip of the microdialysis probe [53]. Hereafter the rat was decapitated and the brain was removed in order to determine the probe position. Immediately after removal the brain was placed in saline stored in a conventional fridge. Crushed ice was added to the saline; the brain was left in this environment for 3 minutes. The brain was then transferred to a pre-chilled Kopf® PA 001 brain blocker and placed in a freezer (-20°C) overnight to freeze. This brain blocker was developed to be used with the stereotaxic atlas of the rat brain developed by Paxinos, and Watson [54]. On the next day successive sections were made (starting anteriorly moving in a posterior direction) with a single sided razor blade in the brain blocker until the stain could be identified. See Figure 3-2 for an example (cresyl violet stain indicated by blue oval).

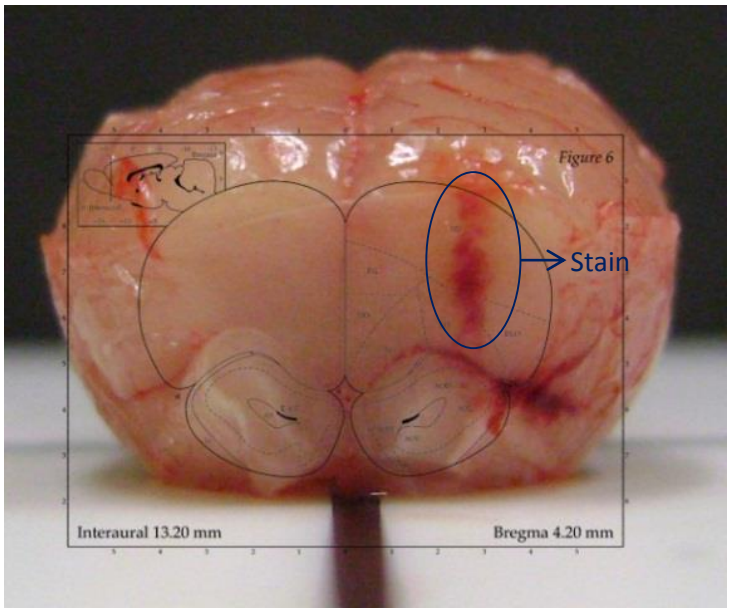

Figure 3-1: Verification of probe position by means of cresyl voilet staining of the probe tract

3.2.6 HPLC analysis of monoamines

NE, DA and its end-stage metabolite homovanillic acid (HVA), 5-HT and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) were determined in microdialysis samples collected from the FSL rat prefrontal cortex. High performance liquid chromatography (HPLC) with electrochemical detection was used to analyse monoamine concentrations in microdialysis samples. Samples were injected into an Agilent 1200 series HPLC equipped with, an

isocratic pump and autosampler set to a flow rate of 0.1 ml/min and injection volume of 20 µl, and coupled with a Coulochem III Electrochemical detector with a coulometric (5014B Dionex Microdialysis Cell by Thermo Scientific) flow cell . The MDTM mobile phase (ESA Inc.) was used (75 mM sodium dehydrogenate phosphate (monohydrate), 1.7mM 1-octanesulfonic acid (sodium salt), 100 ml/l triethylamine, 25 µM EDTA, 10% acetonitrile, with pH adjusted to 3.00 with phosphoric acid) pumped through the Kinetix 2.6µm C18, 100 Å, 150 x 4.6 mm (Phenomenex, Torrance, CA) column. NE, DA and its end-stage metabolite HVA, 5-HT and its metabolite 5-HIAA chromatographs were identified by comparison with elution times of reference standards.

Samples were collected in 100 µl HPLC glass inserts (Agilent, Germany) placed in the refrigerated microfraction collector. 5µl of a solution containing sodium metabisulphite (0.5 mM), ethylenediaminetetra-acetic acid disodium salt (0.3 mM), 60% perchloric acid solution (0.25 M), and a 1500 ng/ml solution of isoproterenol hydrochloride obtained from Merck, Midrand, was added to each vial before sampling was initiated [55]. The Isoproterenol hydrochloride served as the internal standard. The inserts were placed into amber HPLC vials and then transferred to the autosampler. The instrument's software was programmed to inject 20 µl onto the HPLC column, and analysed NE and 5-HIAA and DA and HVA at testing electrode 2 set at +220mV. Results were expressed as ng/ml and converted to nM prior to data analysis.

3.2.7 Data Analysis

All statistical analyses were done with guidance by the Statistical Consultation Service of the North-West University. The data are represented as means and standard error of the mean (S.E.M.). A p-value of less than 0.05 was considered statistically significant. Unpaired two tailed Student's T-tests were performed to compare the different treatment or stressed versus unstressed groups with each other. GraphPad Prism® version 5.00 for Windows (GraphPad Software, San Diego California USA) was used for statistical analysis and graphical presentations of the corticosterone and behaviour data. Two-way ANOVA analyses with group factor and time factor as repeated measure were performed on the microdialysis data of the monoamine levels between -60 to 0 min before the swim stress and again between 20 and 240 min after the swim stress.

3.3 Results

3.3.1 Corticosterone stress response

Figure 3-3 depicts the plasma corticosterone levels in FSL rats on ND+60 before and after the 10 minute forced swim stressor.

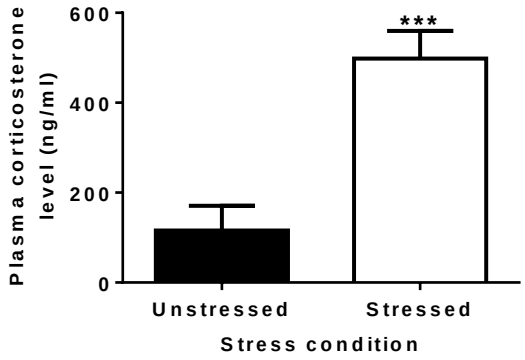

Figure 3-2: Plasma corticosterone levels in FSL rats on ND+60 before and after the 10 minute forced swim stressor, as measured with HPLC. Data points represent the mean ± S.E.M., with n = 7 rats per group and ***p<0.001 (Student's t-test).

It can be seen in Figure 3-3 that exposure to the acute forced swim stress caused a significant increase (>4-fold) in plasma corticosterone concentrations (115.6 ± 55.3 vs 498.1 ± 61.5, $p < 0.001$).

3.3.2 Behaviour

Figure 3-4 depicts the behaviour of FSL rats in the FST and OFT[3].

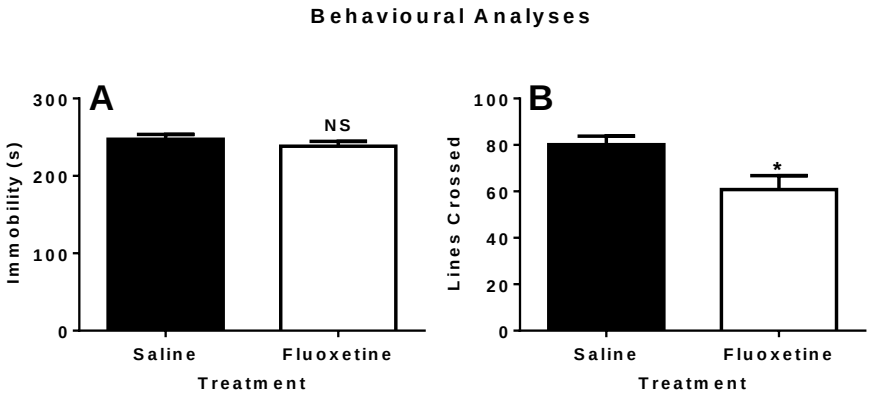

Figure 3-3: (A) Immobility data of FSL rats in the FST after treatment with saline or fluoxetine. (B): Locomotor data of FSL rats in the OFT after treatment with saline or fluoxetine. Data points represent the mean and S.E.M., n=15 rats per group (Student's t-test). s= seconds, NS= not significant, *p<0.05 (Student's t-test)

It can be seen in Figure 3-4A that saline-and fluoxetine-treated rats did not show any significant differences in scored behaviours in the FST. Figure 3-4B shows significant decrease in locomotor activity in fluoxetine-treated FSL rats compared to saline-treated rats (60.80 ± 5.962 vs. 80.13 ± 3.664, $p<0.05$).

3.3.3 Monoaminergic stress response

Figure 3-5 depicts the indicated monoamine concentrations in the prefrontal cortex of FSL rats following saline or fluoxetine treatment before, during and after the 10 minute forced swim stressor.

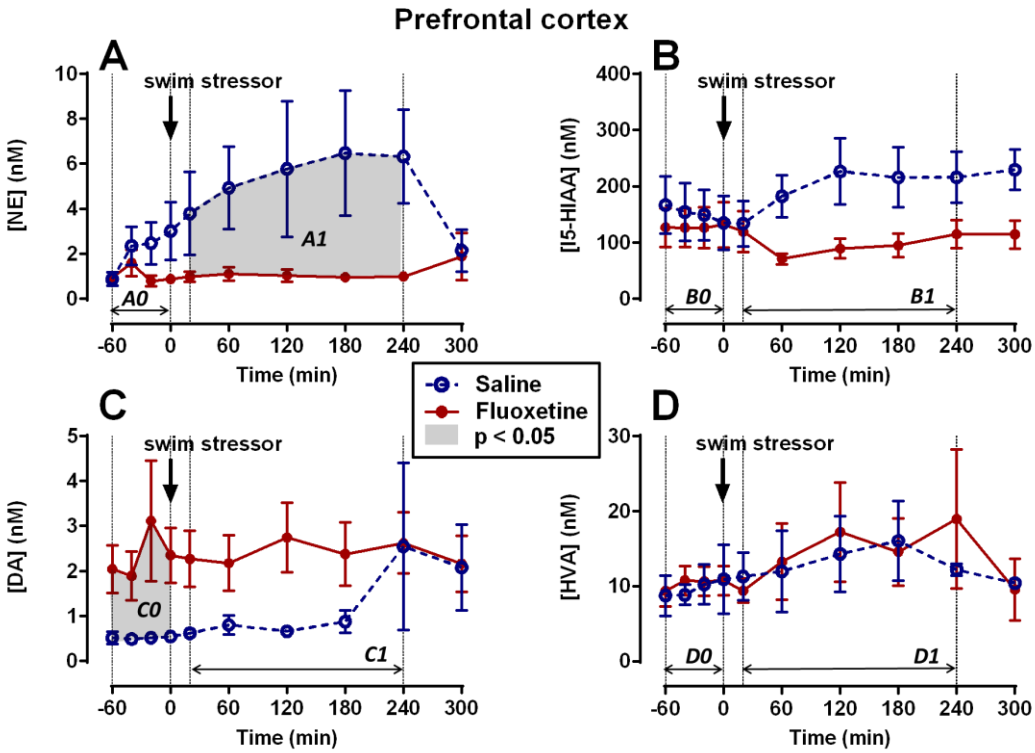

Figure 3-5: Monoamine concentrations in the prefrontal cortex of fluoxetine treated vs. saline treated FSL rats. (A) [NE]= concentration norepinephrine, (B) [5-HIAA]= concentration 5-hydroxyindole-3-acetic acid, (C) [DA]= concentration dopamine, (D) [HVA]= concentration homovanillic acid. Data points represent the mean ± S.E.M., with n=5 rats in the saline group and n=7 in the fluoxetine group. Shaded areas indicate statistically significant differences ($p < 0.05$) as analysed with two-way ANOVA analyses with group factor and time factor as repeated measure.

In Figure 3-5 two-way ANOVA analyses with group factor and time factor as repeated measure were performed on the data of the indicated monoamine levels (after saline or fluoxetine treatment) between -60 to 0 min before the swim (represented by shaded area C0) and again between 20 and 240 min after the swim (represented by shaded area A1).

In Figure 3-5A, area A0 there was no significant interaction between saline- and fluoxetine-treated animals ($F [2, 23] = 1.42$, $p = 0.2613$). There was no significant group effect ($F [1, 23] = 2.62$, $p = 0.1194$), indicating that the NE levels of saline- (1.93 ± 0.39) and fluoxetine-treated (1.19 ± 0.33) animals were not statistically significantly different before the swim stressor. In Figure 3-5A, area A1 there was no significant interaction between saline- and fluoxetine-treated animals ($F [4, 37] = 0.45$, $p = 0.7732$). There was a significant group effect ($F [1, 37] = 13.67$, $p = 0.0007$), indicating that NE levels in the prefrontal cortex were significantly higher in saline-treated animals (5.27 ± 0.98 nM) than in fluoxetine-treated animals (1.33 ± 0.81 nM).

In Figure 3-5B, area B0 there was no significant interaction between saline- and fluoxetine-treated animals ($F [2, 24] = 0.14$, $p = 0.87$). There was no significant group effect ($F [1, 24] = 2.75$, $p = 0.1105$), indicating that the 5-HIAA levels of saline- (126.62 ± 36.53) and fluoxetine- (178.10 ± 39.28) treated animals were not statistically significantly different before the swim stressor. In Figure 3-5B, area B1 there was no significant interaction between saline- and fluoxetine-treated animals ($F [4, 37] = 0.69$, $p = 0.6021$). No significant group effect was present ($F [1, 37] = 3.13$, $p = 0.0853$), indicating that 5-HIAA levels in the prefrontal cortex were not significantly higher in saline-treated animals (183.33 ± 37.4214 nM) than in fluoxetine-treated animals (129.56 ± 34.0482 nM) after the swim stressor.

In Figure 3-5C, area C0 there was no significant interaction between saline- and fluoxetine-treated animals ($F [2, 24] = 0.45$, $p = 0.6435$). There was a significant group effect ($F [1, 24] = 8.83$, $p = 0.0066$), indicating that DA levels in the prefrontal cortex were significantly higher in fluoxetine-treated animals (0.40 ± 0.57 nM) than in saline-treated animals (2.35 ± 0.49 nM). In Figure 3-5C, area C1 there was no significant interaction between saline- and fluoxetine-treated animals ($F [4, 37] = 1.21$, $p = 0.3222$). There was no significant group effect ($F [1, 37] = 3.933$, $p = 0.0549$), indicating that DA levels in the prefrontal cortex were not significantly higher in fluoxetine-treated animals (0.96 ± 0.74 nM) than in saline-treated animals (2.38 ± 0.64 nM) after the swim stressor.

In Figure 3-5D, area D0 there was no significant interaction between saline- and fluoxetine-treated animals ($F [2, 24] = 0.65$, $p = 0.5330$). There was no significant group effect ($F [1, 24] = 0.84$, $p = 0.3683$), indicating that HVA levels in the prefrontal cortex were not significantly higher in saline-treated animals (9.36 ± 8.46 nM) than in fluoxetine-treated animals (19.55 ± 7.15 nM) before the swim stressor. In area D1 (Figure 3-5D) there was no significant interaction between saline- and fluoxetine-treated animals ($F [4, 32] = 0.17$, $p = 0.9543$). There was no significant group effect ($F [1, 32] = 0.06$, $p = 0.81$), indicating that HVA levels

in the prefrontal cortex were not significantly higher in saline-treated animals (15.17 ± 3.56 nM) than in fluoxetine-treated animals (15.88 ± 3.11 nM) after the swim stressor.

3.4 Discussion

3.4.1 Corticosterone stress response

The significant increase in plasma corticosterone levels of rats exposed to the acute forced swim stressor, as compared to the unstressed controls (Figure 3-3), suggest that the stressor induces a significant biological stress response. Furthermore, the data also confirms that the physiological stress response via the HPA-axis remained intact in rats after guide cannula placement surgery and exposure to halothane for microdialysis probe placement.

3.4.2 Developmental effects of pre-pubertal fluoxetine administration on behaviour

3.4.2.1 Depressive like behavior

No significant differences were seen between fluoxetine and saline-treated FSL rats with regard to time spent immobile in the FST (Figure 3-4). The data would suggest that pre-pubertal fluoxetine does not affect depressive-like behaviour in early adulthood. However, immobility data should be interpreted together with locomotor data, as discussed below.

3.4.2.2 Locomotor activity

Pre-pubertal fluoxetine significantly reduced locomotor activity in FSL rats, relative to saline-treated animals (Figure 3-4). The 5-HT_{1A} [56, 57] and 5-HT_{2A} [58, 59] receptor activation is known to increase locomotor activity, whereas 5-HT_{2C} receptor activation has been shown to decrease locomotor activity [58, 59, 60]. Developmental changes induced by fluoxetine in either or both of these systems may be related to the reduced locomotor activity seen in the open-field. An increased 5-HT_{2C} receptor expression or a decrease in 5-HT_{1A} and/or 5-HT_{2A} receptor expression relative to each other may explain these effects.

Reduced locomotor activity in fluoxetine-treated animals would be expected to enhance immobility in the FST. However, this was not observed. We postulate as working hypothesis for further evaluation that pre-pubertal fluoxetine may have induced marginal anti-depressant-like behaviour in early adulthood, but that this response was blunted (masked) by the simultaneous reduction in locomotor activity.

3.4.3 Developmental effects of pre-pubertal fluoxetine administration on monoaminergic stress response

It has been shown in previous rat microdialysis studies that stress (acute, and chronic) increases 5-HT [61], DA and NE [62] concentrations in the prefrontal cortex. The microdialysis data indicates that the pre-pubertal fluoxetine caused a significant attenuation of the NE release in response to an acute stress when compared to untreated rats (Figure 3-5A). This developmental effect can be interpreted as positive since pre-pubertal fluoxetine may prevent an excessive noradrenergic stress response which may avert long-lasting disruptions of noradrenergic functions. It is however also possible that pre-pubertal fluoxetine administration has maladaptive effects and has caused long-term disruptions in the normal noradrenergic stress response. This may prevent the treated subjects from responding appropriately to environmental stressors later in life.

Although our data did not reach statistical significance (probably related to a relatively low number of animals used), there was a trend for a blunting of the serotonergic response in fluoxetine-treated animals (Figure 3-5B). This response was delayed for more than 20 minutes, different from the noradrenergic response. Though not significant the serotonergic stress response data suggests a similar adaptation has occurred in the serotonergic system as described for the noradrenergic system, with similar implications for the positive or maladaptive nature of these developmental changes. This data is however inferred from 5-HIAA since 5-HT could not be reliably measured in our experiments.

Pre-pubertal fluoxetine treatment appears to have significantly increased baseline DA release when compared to saline control rats in the absence of acute stress (Figure 3-5C). The dopaminergic stress response also appears to be attenuated in these rats. In saline-treated animals the dopaminergic stress response seems to be delayed. Saline control rat DA concentrations increased between 180 minutes and 240 minutes after the swim stress. At 240 min after the swim stress saline-treated rats and fluoxetine treated rats showed approximately the same concentrations of released DA. No dopaminergic stress response was apparent in fluoxetine-treated animals. In a review of depression, stress, and anhedonia by Pizzagali (2014) it is stated that a decrease in DA transmission is associated with depression [63]. He also states that direct evidence of decreased DA release is lacking [63]. The increase in DA neurotransmission in the prefrontal cortex of FSL rats treated during pre-puberty may therefore represent a positive developmental adaptation with regard to depressive-like behaviour. The lack of a dopaminergic stress response is however concerning since it may represent, as is the case for noradrenergic and serotonergic neurotransmission, a maladaptive response which prevents appropriate responses to

environmental stressors later in life. The lack of significant differences in HVA concentrations (Figure 3-5D) suggests that the developmental change may have occurred within the re-uptake machinery of the dopaminergic neurons. It has been shown that NET and DA transporters (DAT) have the ability to transport both NE and DA [64]. These changes might therefore have occurred in the expression and distributon of DAT and NET in the brain resulting in increased DA concentrations in the prefrontal cortex of fluoxetine-treated FSL rats. Non-specific transporters known as organic cation transporters (OCTs) are also present in the brain [65, 66]. Three subtypes exist and are found in the brain [67, 68, 69], the third of which has been shown to be inhibited by corticosterone and may therefore result in increased brain monoamine concentrations under conditions of stress [66, 67]. Since the corticosterone concentrations were shown to be significantly elevated in FSL rats exposed to a 10 minute forced swim stress (see 3.3.1 and 3.4.1) changes might have occurred in the expression and distributon of OCTs in the brain resulting in reduced DA reuptake and an increase in DA Concentration in the prefrontal cortex of fluoxetine-treated FSL rats.

According to Kvetnansky and colleagues (2009), acute stress causes brief activation of the HPA axis as a result of alterations to catecholamine (CA)[4] systems such as NE and DA neurons in the brain [27]. After cessation of the stressor, homeostasis is usually restored. However the nature and intensity of the stressor are factors which may determine whether normal CA functioning is restored. During stress sustained over long periods of time the synthesis of CAs may not meet the demand placed on the system resulting in reduced CA levels available for release at the synapse [27]. Reuter and Jacobs (1997) reviewed the evidence of the effect of stressors of various types on extracellular 5-HT concentrations [28]. They reported that 5-HT concentrations increase by 30-100% following the introduction of acute stress in virtually all the brain areas studied [28].

3.5 Conclusions

To our knowledge the data presented here is the first evidence of significant developmental effects of pre-pubertal fluoxetine administration on monoaminergic stress responses which may affect individuals with a predisposition towards the development of MDD or those suffering from MDD to cope successfully with acute environmental stressors later in life. Significant changes were also observed in locomotor activity between fluoxetine treated FSL rats and saline control rats. Targets for further research to explain the processes responsible for the molecular changes observed in this study were also identified. Specific research topics suggested include studies designed to explore the role of fluoxetine in the expression and distribution of the serotonergic receptor subtypes (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}), the development and distribution of monoamine transporters (DAT, NET, and OCTs), as well as

the mechanisms by which serotonin might influence and regulate the development of the noradrenergic and dopaminergic neurodevelopment.

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Chapter 4 Summary, conclusions and recommendations

In order to provide a comprehensive overview of the study as a whole, the results and discussion (chapter 3 and addendum A) will be summarised and further discussed in this chapter, reconciling the study outcomes with the study objectives, and ultimately culminating in final conclusions and recommendations for the study as a whole.

4.1 Summary of results

The current study demonstrated a statistically significant decrease in core body temperature at 15, 30, and 60 minutes after injection of 8-OH-DPAT in FSL rats relative to FRL rats (table 4-1).

Table 0-A: Summary of data of core body temperature changes at 0, 15, 30, and 60 minutes after 8-OH-DPAT injection (refer to addendum A). The directions of the arrows indicate decreases (downwards), and no changes (horizontal). Red arrows indicate statistically significant decreases.

Central temperature: rat strains relative to each other	
Time after Injection	**Temperature of FSL rats relative to FRL rats**
0 min	⟷
15 min	↓
30 min	↓
60 min	↓

A statistically significant increase in corticosterone concentrations were found in FSL rats exposed to a 10 minute forced swim stress relative to unstressed control FSL rats (table 4-2).

Table 4 2: Data summary of Corticosterone concentration differences of unstressed and stressed groups relative to each other (refer to chapter 3). The direction of the arrow indicates increases (upwards) changes. Red arrow indicates a statistically significant increase.

Corticosterone levels of groups relative to each other	
Analyte	**Corticosterone levels of stressed FSL rats relative to unstressed FSL rats**
Corticosterone	↑

The monoaminergic stress response (sans treatment) data could not be interpreted meaningfully due to the renovations described in addendum A.

The current study demonstrated that pre-pubertal fluoxetine treatment from ND+21 to ND+34 had significant developmental effects on FSL rat behaviour in the OFT in terms of locomotor activity on ND+60. A summary of the behavioural findings is provided in table 4-3 below.

Table 4-3: Data summary of the behavioural effects observed in treatment groups relative to each other (refer to chapter 3 and addendum A). The directions of the arrows indicate decreases (downwards), and no changes (horizontal), where a red arrow indicates statistically significant change.

Behavioural changes of groups relative to each other		
Behaviour	**Vehicle**	**Fluoxetine**
Forced Swim Test Data		
Immobility	↔	↔
Swimming	↔	↔
Climbing	↔	↔
Open Field Test Data		
Locomotor activity	↔	↓
Anxiety-like	↔	↔

The current study also demonstrated that pre-pubertal fluoxetine treatment (between ND+21 and ND+34) had significant neurodevelopmental effects on the noradrenergic and dopaminergic neurotransmitter systems as measured in microdialysis samples collected from male FSL rats between ND+60 and ND+62. Though not statistically significant the results of 5-HIAA concentrations suggest neurodevelopmental changes also occurred in the serotonergic system. A summary of the differences in the monoaminergic stress response between fluoxetine-treated and saline-treated male FSL rats are given in table 4-4.

Table 4-4: Monoaminergic stress response data summary of treatment groups relative to each other (refer to chapter 3). The directions of the arrows indicate increases (upwards), and no changes (horizontal). Red arrows indicate statistically significant increases.

Monoamine concentrations : goups relative to each other				
Treatment Group	**Vehicle**	**Fluoxetine**	**Vehicle**	**Fluoxetine**
Analyte	**Before Swim Stress**		**After Swim stress**	
NE	↔	↔	↑	↔
5-HIAA	↔	↔	↑	↔
DA	↔	↑	↔	↑
HVA	↔	↔	↔	↔

A summary of the changes from baseline in the monoamine concentrations for each analyte after the swim stress are given for FSL rats not exposed to stress or FSL rats exposed to a 10 minute swim stress in table 4-5.

Table 4-5: Summary of monoamine concentration changes (without pre-pubertal drug treatments) in response to the swim stress for each analyte (refer to addendum A). The directions of the arrows indicate increases (upwards), decreases (downwards), and no changes (horsizontal).

Monoamine concentration changes: within groups		
Time interval	**After swim**	
Group	**Unstressed**	**Stressed**
NE	↓	↑
5-HIAA	↑	↑
DA	↑	↑
HVA	↑	↓

4.2 Final Discussion and Conclusions

FSL rats display muscarininc and serotonergic supersensitivity relative to control FRL rats: FSL rats displayed statistically significant exaggerated hypothermic responses to 8-OH-DPAT injection at 15, 30, and 60 minutes. Both cholinergic and serotonergic challenges have been used in order to ensure the integrity of the FSL and FRL rat lines

(Overstreet *et al*., 2005). This is necessary due to the fact that the animals in both strains are more than 90% inbred and genetic drift may occur between different breeding colonies in laboratories around the world (Overstreet *et al*., 2005).

Decreased locomotor activity in the OFT in fluoxetine-treated FSL rats relative to saline-treated FSL rats: The decreased locomotor activity in the OFT in fluoxetine-treated FSL rats relative to saline-treated FSL rats observed in the study may be explained by the effects of serotonin receptor subtypes. It has been reported that 5-HT_{1A} receptor activation causes an increase in locomotor activity (Gualda *et al*., 2011; Mignon & Wolf, 2002). This has also been reported for the 5-HT_{2A} receptor subtype (Halberstadt *et al*., 2009; McOmish *et al*., 2012). Furthermore, 5-HT_{2C} receptor activation has been shown to decrease locomotor activity (Halberstadt *et al*., 2009; Martin *et al*., 1998; McOmish *et al*., 2012). Developmental changes induced by fluoxetine in either or both of these systems may be responsible for the reduced locomotor activity seen in the open-field. An increased 5-HT_{2C} receptor expression or a decrease in 5-HT_{1A} and/or 5-HT_{2A} receptor expression relative to each other may explain these effects. The decrease in locomotor activity in fluoxetine-treated rats may have also been responsible for masking a significant improvement in depressive like behaviour of these rats in the FST.

Acute forced swim stress on ND+60 induces a significant stress response in FSL rats: A significant biological stress response, as signified by a statistically significant increase in plasma corticosterone levels was observed in rats exposed to the acute forced swim stress relative to unstressed control rats. Furthermore, the data also confirms that the physiological stress response via the HPA-axis remained intact in rats after guide cannula placement surgery and exposure to halothane for microdialysis probe placement.

Increased noradrenergic stress response in fluoxetine-treated animals relative to saline-treated animals: Pre-pubertal fluoxetine treatment caused decreased NE concentrations in the prefrontal cortex of fluoxetine-treated rats after exposure to an acute swim stress. This suggests that pre-pubertal fluoxetine treatment caused attenuation of norepinephrine release in response to the acute stressor in early adulthood. A change such as this may be beneficial if it prevents an exaggerated stress response which may have many lasting negative effects (Mamalaki *et al*., 1992; Rusnák *et al*., 2001; Watanabe *et al*., 1995) potentially leading to the development of MDD. However, it should also be considered that such a change could lead to maladaptive responses to environmental stressors. This possibility should be considered since an increase in NE release is normally associated with exposure to stress (Kvetnansky *et al*., 2009). Though not significant, it was also observed

that there was a trend for 5-HIAA concentrations to be reduced in fluoxetine treated animals when compared to saline-treated rats, following the forced swim stressor. Similar conclusions could be inferred from this data for the serotonergic system as those drawn from the NE data (Rueter & Jacobs, 1997). With refinement of the analysis technique it might be possible to demonstrate similar changes for 5-HT concentrations.

Increased DA in fluoxetine-treated rats relative to saline control rats at baseline: A significant increase was seen in baseline DA concentrations, before the swim stress in fluoxetine treated FSL rats when compared to saline treated FSL rats. This would suggest long-term fluoxetine-induced enhancement of dopaminergic neurotransmission. Since decreased DA concentrations are associated with MDD (Pizzagalli, 2014) this may be advantageous in decreasing depressive-like behaviour later in life. After the stressor, at 180 minutes, the saline-treated rats showed a far more pronounced dopaminergic stress response than fluoxetine-treated rats. Following the acute stressor, this difference became insignificant and ultimately vanished after 240 minutes. These changes may also be maladaptive, as described for both NE and 5-HIAA, since no dopaminergic stress response appears to be apparent in fluoxetine-treated FSL rats and (much like the other monoaminergic systems) an increased secretion in response to a stressor is expected for DA (Mamalaki *et al*., 1992; Rusnák *et al*., 2001; Watanabe *et al*., 1995).

No significant differences in HVA concentrations in fluoxetine-treated and saline-control rats relative to each other before and after exposure to an acute swim stress: It was also interesting to note that HVA concentrations did not increase in fluoxetine-treated rats when compared to saline-treated controls. One would expect a proportional increase in HVA concentrations to the increase in DA concentration observed in these rats, since it is the end-stage metabolite of DA (Kvetnansky et al., 2009). The three classes of transporters which are responsible for DA reuptake are DAT, NET and OCTs (Torres *et al*., 2003; Amphoux *et al*., 2006; Wu *et al*., 1998). It is interesting to note that OCT type 3 is inactivated by corticotropin-releasing hormone (Gasser *et al*., 2006; Wu *et al*., 1998). This hormone is intricately involved in modulating stress responses in animals via its effect on the HPA axis (Belmaker & Agam, 2008). These changes suggest a role for OCT type 3 in increasing monoamine concentrations in the brain in response to elevated corticosterone release. Pre-pubertal fluoxetine administration may therefore have resulted in changes to these transport proteins (NET, DAT, and OCTs), which prevented DA reuptake in fluoxetine treated rats. Changes may also have occurred in the HPA axis resulting in inhibition of OCT type 3 functioning specifically due to increased circulating corticotropin-releasing hormone.

No statistical differences were seen in anxiety-like behaviour as measured in the OFT between fluoxetine-treated FSL rats and saline-treated FSL rats: The lack of significant changes observed in anxiety-like behaviour in FSL rats treated with pre-pubertal fluoxetine when compared to saline controls can be explained by the fact that increased anxiety-like behaviour is not associated with FSL rats (Neumann et al., 2011; Overstreet et al., 2005). In fact, the FSL rat is considered by some to be a selective animal model for depression (Overstreet, 1993).

No statistical differences were seen in swimming and climbing behaviour as measured in the FST between fluoxetine-treated FSL rats and saline-treated FSL rats: No significant changes were seen in swimming and climbing behaviours in the FST in FSL rats treated with pre-pubertal fluoxetine when compared to saline controls. Increases in one or both of these behaviours may have been masked due to the decreased locomotor activity discussed above.

No meaningful results were obtained in the monoaminergic stress response study (no treatment): The most likely explanation for the fact that the data generated in this study was rendered unusable is that a considerable amount of stress was caused in the stress sensitive FSL rats used in the study by unexpected renovations to a building adjacent and connected to the one housing the microdialysis laboratory at North-West University.

Final conclusions and discussion of the relevance of the study to children and adolescents at risk of or suffering from MDD: The role of stress in the aetiology of MDD is a field of active research with many questions still unanswered. Any developmental changes which improve or impair individuals at-risk of or suffering from MDD's chances of successfully coping with environmental and psychological stressors require thorough investigation. In this study we demonstrated that early-life exposure to fluoxetine, one of two drugs approved for treating MDD in children and adolescents, causes long-term developmental changes which alters how the subjects respond to stress. These findings expand evidence about the potential interplay between features associated with the monoaminergic, the hypothalamic-pituitary-adrenal axis hyperactivity, and neurodevelopment/neuroplasticity hypotheses of depression (discussed in 2.4.2, 2.4.3, and 2.4.4) (Ehlert *et al*., 2001; Leonard, 2001; Schiepers *et al*., 2005). Though further studies are needed, if expanded upon, these findings may potentially change the way in which MDD is treated. Should the changes described here prove to be beneficial a strong argument can be forwarded for the promotion of preventive fluoxetine treatment for children and adolescents at risk of developing MDD later in life due to stressful environmental conditions (such as

growing up in a war-torn country). If the changes described here prove to be harmful new strategies to treat MDD in children and adolescents are urgently needed since fluoxetine is one of only two drugs approved by the FDA for this purpose.

4.3 Recommendations

This study successfully addressed the objectives described in chapter 1. We successfully showed that pre-pubertal development of the monoaminergic systems in the prefrontal cortex of male FSL rats are vulnerable to fluoxetine exposure as observed in microdialysis experiments conducted in early adulthood. This study allowed us to implement and validate the microdiaylsis technique for measuring monoamines in rodents. We also demonstrated that pre-pubertal fluoxetine exposure affects the locomotor activity measured in early adulthood. Despite the success reported it should be kept in mind that significant limitations are present in the study. Several questions raised by the current study require further investigation. Accordingly the following recommendations are made:

1. Limitations of the study and possible ways to improve the study:
    a. The inclusion of a control strain of rats, namely the FRL rat, would greatly aid in the interpretation of the findings of the current study. This may illuminate the role of a genetic predisposition towards excessive stress responses and MDD to altered monoaminergic stress responses. This would also provide one means by which it would be possible to ascertain whether the changes described in monoaminergic release in the current study are beneficial. The results of such a study may, for instance, show whether fluoxetine-treated FSL rats display monoaminergic stress responses which more closely resemble untreated FRL rats than untreated FSL rats.
    b. The monoaminergic stress response data of untreated rats were rendered uninterpretable, due to additional stress caused by the facility renovations discussed in addendum A. These data may aid in the interpretation of the developmental changes observed in FSL rats treated with fluoxetine during pre-puberty and should therefore be repeated.
    c. The statistical power of microdialysis studies was insufficient, due to relatively low FSL rat birth rate, which resulted in decreased availability of experimental animals. Given the trends for some of the monoaminergic studies (e.g. the 5-HIAA stress response study) significant changes may be found if the statistical power of the studies is improved by increasing the number of subjects in each treatment group.

2. Studies should be designed to further investigate the decrease in locomotor activity observed in male FSL rats exposed to pre-pubertal fluoxetine treatment. These studies should be designed in order to ascertain whether and how 5-HT receptor subtypes are involved in the developmental change reported here:
    a. Multiple experimental approaches should be used in such studies. Examples of such studies would include acute administration of agonists and antagonists specific for 5-HT receptor subtytypes 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} before the FST and after fluoxetine treatment as described in the current study. These studies will allow us to identify which, if any, of these receptor subtypes are involved with the decreased locomotor activity observed in this study.
    b. Studies should be designed to investigate the distribution and density of these 5-HT receptor subtypes in untreated and treated (between ND+21 and ND+34) FSL rats to identify the specific brain regions involved in the decreased locomotor activity observed in FSL rats treated with fluoxetine during pre-puberty.
    c. It is important to compare the effect of fluoxetine to that of antidepressants from other classes, in particular to understand the importance of various systems involved in neurodevelopment.
3. Given the role of stress in various hypotheses of the aetiology of MDD further studies about the beneficial and maladaptive developmental changes observed in fluoxetine-treated rats are essential.
    a. Further studies could follow the same treatment protocol but instead of an acute stressor a chronic one may be employed to more accurately reflect what is seen in humans with MDD. This would also allow us to investigate markers which change over a longer time frame. A neurodevelopmental marker such as BDNF upregulation is such an example.
    b. Immunohistochemical studies should be conducted to explore the developmental effects of pre-pubertal fluoxetine exposure on monoamine transporters in terms of distribution and regional density as well as the development and anatomical distribution of the specific monoaminergic pathways.
    c. Studies which concurrently investigate the monoaminergic stress response and biomarkers associated with other hypotheses of the aetiology of MDD should be designed. Since tryptophan is the common precursor for 5-HT and 5-HIAA and products of the kynurenine pathway (described in 2.4.6 above) a

study may be designed to investigate the effect of pre-pubertal fluoxetine administration on 5-HT and 5-HIAA concentrations in the brain as well as markers associated with the kynurenine pathway. This study could also be expanded to examine the neuroprotective and damaging effects of the products of the kynurenine pathway. It is also known that cell mediated immunological processes upregulate indoleamine 2,3-dioxygenase, the enzyme responsible for tryptophan metabolism in the kynurenine pathway and this will cause a decrease in 5-HT production (described in 2.4.6). Multi-modal studies such as this hold great promise in illuminating the dynamic and complex interplay of these various systems with each other and could lead to new drug targets for treatment of MDD.

d. Further studies on the developmental effects of pre-pubertal fluoxetine exposure on the HPA axis and its interplay with OCT type 3 should be conducted to further illuminate the role of chronic and acute stress in the development of MDD.

Adendum A: Additional material

A.1 Materials and Methods

A.1.1 Subjects

A total of 74 Flinders sensitive line (FSL) and Flinders resistant line (FRL) male rats were used in the study. All experiments were approved by Ethics Committee of North-West University (ethical approval numbers: NWU-00045-10-5S and NWU-0028-08-A5) The rats were pair–housed in the North-West University Vivarium with the environmental temperature and humidity maintained at 22 ± 1°C and 50% respectively. A 12:12-h light/dark cycle (lights on 06:00 to 18:00) was followed and food and water were available *ad libitum*. On ND+57 the rats used for *in vivo* microdialysis and corticosterone assays were moved to the microdialysis lab. The same light/dark cycle and temperature was maintained in this lab, whereas rats for behavioural analyses were housed in the vivarium until testing on ND+60.

Prior to the commencement of the study, 18 rats (9FSL rats and 9FRL rats) were used to determine whether the integrity of the FSL rat strain was still intact. It was deemed necessary to do so due to upheavals caused by necessary renovation of the North-West University vivarium. This was accomplished by comparing the hypothermic response to a single 8-hydroxy-2-(di-n-propylpamino) tetralin (8-OH-DPAT) injection (as discussed in A.1.3) in 9 FSL rats with 9 Flinders resistant-line (FRL) rats. Male FSL rats were used exclusively in the rest of the study.

A.1.2 Drug Administration and Dosages

Saline (vehicle) or fluoxetine was administered to rats from ND+21 to ND+34 at a dose of 10mg/kg/day (Gomez *et al*., 2014; Hansen *et al*., 2011). A similar dose was also used in previous studies in our laboratory and elsewhere where the drug was used for its antidepressant-like effect (First *et al*., 2011; Gomez *et al*., 2014; Liebenberg *et al*., 2010; Overstreet & Griebel, 2004; Saenz del Burgo *et al*., 2011).

The parenteral route is preferred due to the predictable response and control over dose. Since chronic intraperitoneal (i.p.) injections are more likely to cause tissue damage and discomfort in smaller rats, the subcutaneous route (s.c.) was implemented. In addition, s.c. administration is predictable and has a bioavailability similar to that of i.p. administration (Steyn, 2011). This simple method also allows for the injection of relatively large volumes and site selection is not as critical as with the i.p. injection method (Nebendahl, 2000). The technique has been used successfully in other studies to effectively deliver fluoxetine and

other antidepressants to the experimental animal (Hansen et al., 2011; Mendes-de-Silva et al., 2002), as well as in our laboratory (Liebenberg et al., 2010; Steyn, 2011).

8-Hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) was administered via s.c. injection in a dose of 0.25mg/kg (Van Zyl, 2008).

For all microdialysis guide cannula placement surgeries (in the prefrontal cortex) anaesthesia was induced by i.p. injection (0.1 ml/100g) of a mixture of xylazine (60mg/kg) and ketamine (10mg/kg) (Kotronoulas et al., 2013). 5 ml of a 100% halothane solution (Halothane-M & B; Safeline Pharmaceuticals) was used to render animals motionless by placing them in an airtight enclosure to facilitate microdialysis probe placement on day 3 following surgery (Visser, 2008).

A.1.3 Induced hypothermic response

9 FSL rats and 9 FRL rats were used to verify that the FSL rat strain housed at the Vivarium still displayed its characteristic serotonergic super sensitivity. Typical validation involves comparison of the hypothermic response of FSL rats versus that of FRL rats to a single administration of 8-OH-DPAT, a potent and selective 5-HT_{1A} agonist. It has been demonstrated by Overstreet and colleagues (1994, 2005) that FSL rats show an exaggerated hypothermic response when compared to FRL rats following injection of 8-OH-DPAT.

A.1.4 Behavioural Studies

30 male FSL rats were divided into two treatment groups. The first group received saline injections as vehicle control, the second group received fluoxetine injections. Treatment took place from ND+21 to ND+34. All behavioural experiments were video-taped and scored by a researcher blinded to the treatment groups.

A.1.4.1 Open Field

A.1.4.1.1 Locomotor Activity

Fatigue and anhedonia are known symptoms of MDD and lead to decreased general activity in the affected individual. In order to test for this phenomenon in rat models of depression locomotor activity is assessed (Golembiowska *et al*., 2012). The apparatus used for the test consisted of a square opaque perspex container. The floor of the box served as a 1 m^2 test arena and was subdivided into sixteen 25 x 25 cm smaller squares (Liebenberg *et al.*, 2010).

In this test the total number of lines crossed within the 5 minute testing session was used as an indication of general activity. This test is also done to test the reliability of the data generated in the FST. If the generalised locomotor activity is increased one cannot interpret reduced immobility in the FST as a decrease in depressive-like behaviour (see A.1.4.2).

A.1.4.1.2 Anxiety

Anxiety is considered to be a prominent comorbid condition frequently encountered in MDD (Overstreet & Wegener, 2013). In order to test whether this MDD associated condition was present in our experimental animals we assessed anxiety-like behaviour in rats. Time spent in the central 4 squares of the OFT enclosure relative to that spent along the walls of the OFT container was measured. It would appear that rats are naturally inclined to avoid well illuminated open spaces. It is therefore thought that a rat displays more anxiety-like behaviour the less time is spent in the central four squares (Overstreet & Griebel, 2004). In this test each rat was placed in the apparatus and was permitted to explore the enclosure freely for a period of 5 minutes under a light intensity of 80 lux (Liebenberg et al., 2010).

A.1.4.2 Forced Swim Test

The forced swim test (FST), first described by Porsolt in 1977, has been used successfully to monitor depressive-like behaviour in rodents for many years (Porsolt *et al*., 1977; Cryan *et al*., 2002). It has since been modified by Lucki and colleagues (1997) in order to distinguish between behaviour mediated by the noradrenergic and serotonergic systems.

The test was conducted in clear perspex cylinders (20 cm diameter and 40 cm high) (Castagni *et al*., 2010). On the day of the test the cylinder was filled to a depth of 30 cm with 25 ˚C water and the test was conducted under a light intensity of 200 lux (Cryan *et al*., 2002; Liebenberg *et al*., 2010). No pre-test was used since this has been proven to be unnecessary when using FSL rats (Overstreet & Griebel, 2004). The rats were placed in the water for 7 minutes. When scoring, the first and last minute were ignored, which means scoring was only done for a 5 minute period (Liebenberg *et al*., 2010). The experiments were recorded by video camera for later scoring. The movements of the rats were scored according to the criteria laid out by Cryan and colleagues (2002). Firstly, immobility is defined as an absence of active movements other than those necessary to keep the animal's head above water. Secondly, swimming movements are defined as any horizontal movement, including those which involve crossing into another quadrant. Lastly, climbing behaviour is defined as any upward-directed movement along the walls of the cylinder away

from the water level (Cryan *et al*., 2002). For a graphic depiction of the expected animal behaviour see Figure A-1.

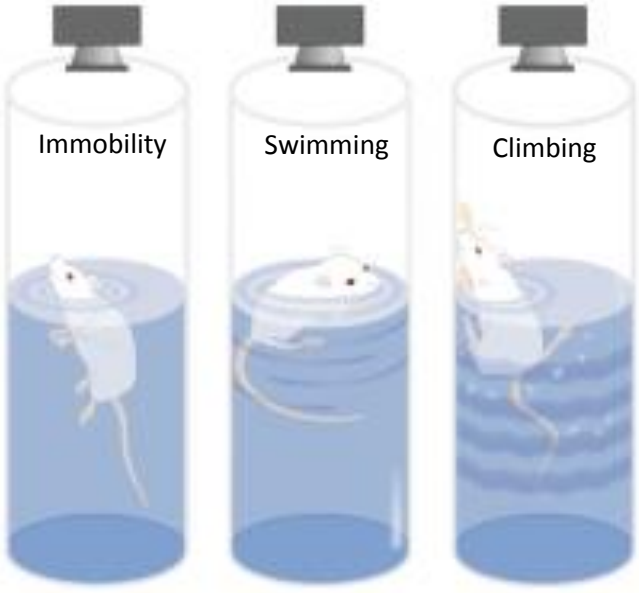

Figure A-1: Illustration of scoring behaviours during the FST (Cryan et al., 2002).

A.1.5 Stress Response

A.1.5.1 Acute forced swim stressor

As mentioned in paragraph 2.9, chronic stress may disturb the balance between catecholamine synthesis and demand, so that less neurotransmitter may be available for release during an acute stressor (Kvetnansky *et al*., 2009). The current study aimed at investigating whether pre-puberty treatment with fluoxetine could alter acute monoaminergic stress response in early adulthood. Previous studies induced stress by means of a single 10 minute forced swim (Purdy *et al*., 1991; Schwartz *et al*., 1987). How corticosterone and monoamine concentrations were used as markers for acute stress in the current study is discussed below. Procedures for the forced swim is similar to those used in the FST (see A.1.4.2).

A.1.5.2 Corticosterone analysis

Corticosterone concentrations were determined from trunk blood after decapitation according to the method described before in 3.2.4.2 (Viljoen *et al*., 2012). A short description follows.

A.1.5.2.1 Preparation of standards

A corticosterone stock solution was created by dissolving 100 µg/ml of corticosterone in 20% methanol. The stock solution was stored in an amber volumetric flask in a refrigerator. Trunk blood collected from FSL rats following decapitation was collected in heparinised tubes and centrifuged at 3000 revolutions pre minute (rpm) for 15 minutes. The plasma fraction from each blood sample was then pooled into a glass beaker and subsequently treated with activated decolorizing carbon to extract the endogenous corticosterone. The suspension created by this procedure was stirred for ±90 minutes at room temperature and pipetted into a glass tube. This glass tube was centrifuged at 3000 rpm for a total of 10 minutes. A 0.45-µm Millipore filter was then used to remove carbon particles from the top layer of plasma. Two sets in the concentration range of 10-500 ng/ml were prepared. The first with distilled water for water standards, the second with activated decolorizing carbon treated plasma for plasma standards.

A.1.5.1.2 Sample preparation

Screw-capped glass tubes (dimension: 10 x 100 mm) were prepared for each sample (water standard or plama standard or test plasma) by adding 50 µl of the internal standard (1µg/ ml Dexamethasone). 500 µl of the sample was added as well as 5 ml of dichloromethane, vortexed for 2 minutes and centrifuged at 3000 rpm for 15 minutes. Removal of the upper layer (either distilled water or plasma depending on which sample was being prepared) followed. This enabled the transferral of the lower organic layer to conical tubes. Nitrogen was used at room temperature to evaporate the liquid from the preparation. 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 autosampler.

A.1.5.1.3 High performance liquid chromatography conditions

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).

A.1.6 Microdialysis

All microdialysis procedures were based on the description of the technique by Visser (2012).

A.1.6.1 Principles and Rationale

Microdialysis is a technique that allows for the *in vivo* monitoring of various chemicals (endogenous and exogenous) within human and animal tissues (Bazzu *et al*., 2012). The technique was used for the first time in 1972 for research purposes (Bazzu *et al*., 2012) and has since been used in numerous studies of neurotransmitter levels in animals under various experimental conditions (Li *et al*., 2006). The underlying principle of the technique is to introduce a probe that mimics a capillary vessel into the extracellular fluid (ECF). The diameter of the probe is small (more or less 1 mm) and contains 2 lumens which can be connected to inlet and outlet tubing respectively. At the tip of the probe a semi-permeable membrane is present (Hillered & Persson, 2003). A physiological fluid such as artificial cerebrospinal fluid (aCSF) is used as perfusate and is pumped into the probe inlet. The anylate is collected from the outlet tube at the same rate as the perfusate is injected into the probe. This ensures that the concentration gradient at the tip of the probe is maintained. At the probe tip endogenous compounds (such as neurotransmitters) dissolved in the aCSF, will enter the probe, moving along their respective concentration gradients, and will exit the probe via the outlet. It is also possible to study the influence of an exogenous compound on the neurochemistry of the brain by adding this compound to the perfusate. The concentration gradient will favour the movement of this molecule into the ECF. This process is called retrodialysis and is very useful since the collection of endogenous compounds is not affected by this procedure (Chaurasia *et al*., 2007).

A.1.6.2 Guide cannula implantation procedures

Stereotaxic surgery was performed in order to place a microdialysis probe in the PFC, an area implicated in MDD as described in paragraph 2.2. A mixture of xylazine (60mg/kg) and ketamine (10mg/kg) was used to induce and maintain anaesthesia. The anaesthetic solution was given intraperitoneally in a dosage of 0.1ml/100g (Kotronoulas *et al*., 2013). Vital signs such as respiratory rate and pattern, cardiac function, and cyanosis of the tongue were monitored throughout the procedure. The body temperature of the subject was maintained at 37 ˚C with the aid of a surgical heating pad.

When the appropriate plane of anaesthesia was reached, the animal was mounted in the stereotaxic frame. The scalp was incised along the midline with a number 10 scalpel blade and extended for 2 cm in order to expose the underlying bone. The periosteum was reflected away from the bone in a lateral direction to enable visualisation of the bregma (pictured in figure A-2) skull suture which was used as zero reference.

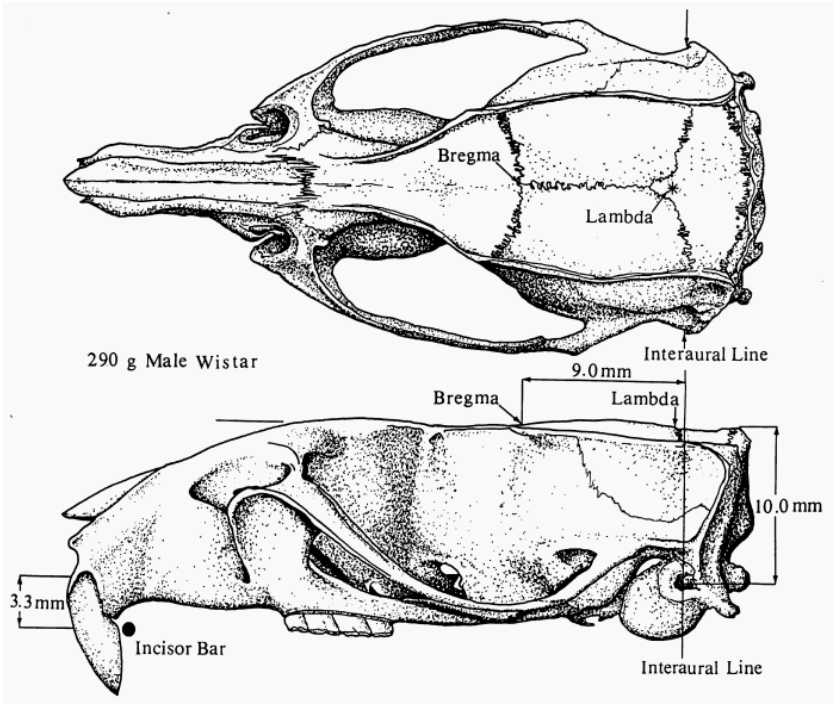

Figure A-2: Anatomy of the rat skull (Paxinos and Watson, 2005)

The skull was disinfected with a drop of 20% hydrogen peroxide solution which also inhibits bleeding and in so doing, enabled better visualisation of the surgical area. A mark was made 4.2 mm anterior-posterior (AP) and 2.4 mm lateral (L) to bregma. The guide cannula was placed, 2.4 mm ventral (V) to bregma, into the PFC once the opening for the probe had been created as depicted in the Figure A-3 (Paxinos & Watson, 2005). A dental drill bit, 1.15 mm in diameter, was used to create the opening for the probe, taking care not to puncture the dura to prevent excessive bleeding. Three smaller holes (0.69 mm in diameter) were then drilled near the opening in order to place jeweller screws, 0.9mm x 1.92mm, shallowly into the bone to provide extra support and anchorage for the cannula without causing undue pressure and irritation of the dura. The dura were then punctured with a sharp, sterile syringe needle prior to lowering the microdialysis probe gently into the opening to the desired depth (2.4 mm ventral). Acrylic dental cement was then used to secure the cannula to the skull and support screws after drying the skull with cotton swabs. An intraperitoneal diclofenac injection, dose 2mg/kg, was given after completion of the surgery for pain control.

The rat was then transferred to the microdialysis cage and left to recover for 3 days prior to implantation of the microdialysis probe and commencement of the microdialysis procedure. The subjects were monitored for signs of pain and infection during the recovery phase and immediately euthanised if any were noted (Visser, 2012).

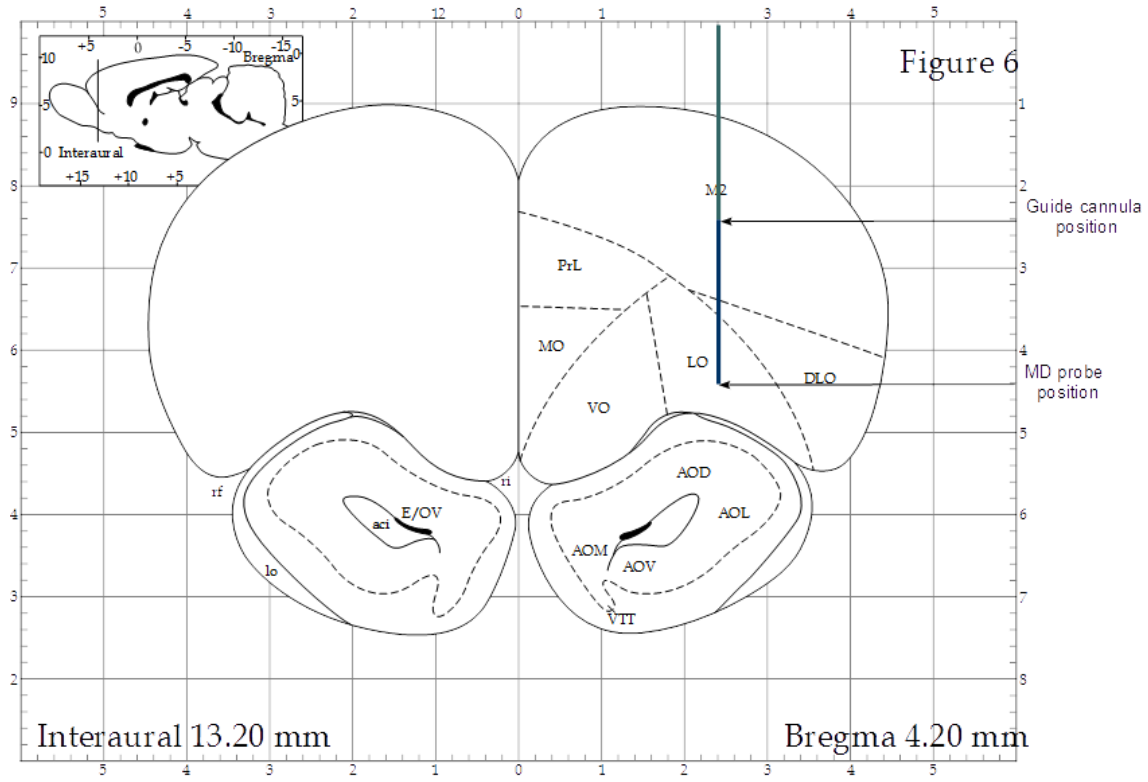

Figure A-3: Probe positioning using stereotaxic atlas (adapted from Paxinos & Watson, 2005; Visser, 2012)

A.1.6.3 Microdialysis probe implantation procedure

On the day preceding the microdialysis sample collection (18:00) the rat was placed in an airtight cage with a halothane soaked cotton swab. As soon as the rat became immobile it was removed and the probe was placed into the guide cannula before the animal recovered full consciousness. The rat was then placed into the microdialysis enclosure and perfusion was initiated at 1.0 µl/min to allow equilibrium to be achieved prior to the microdialysis sampling procedure at 08:00 the next day (Wegener, 2000). The probe was placed on the evening prior to the sampling procedure to reduce a halothane mediated increase or decrease in sample monoamine concentrations. This decision was made based on a study conducted in our laboratory by Harvey and colleagues (2006). They showed that halothane causes an increase in frontal cortex NE concentrations and a decrease in frontal cortex DA concentrations in whole brain tissue. 5-HT concentrations were not affected by halothane exposure (Harvey *et al*., 2006).

A.1.6.4 Sampling Procedure

The samples were collected over a time frame of 7 hours and 20 minutes. Initially the rat was placed in the microdialysis enclosure following connection of the microdialysis probe to the sampling system. Perfusion was initiated at 18:00 on the day before the experiment at a flow rate of 1.0 µl/min. On the day of the experiment sampling was initiated at 08:00. Samples were collected in 20 minute intervals. The first 3 samples were used to determine baseline levels of the monoamines. At the start of the fourth sampling interval the rat was placed in a swimming cylinder filled with water (25°C) to a depth of 30cm at a light intensity of approximately 200 lux (Cryan *et al*., 2002; Liebenberg *et al*., 2010). The rat was removed from the swimming cylinder after a 10 minute acute swim stress and a further 18 samples were collected over a period of 6 hours. Upon removal from the FST cylinder and gently dried with tissue paper and returned to the microdialysis enclosure.Only the first sample collected in each hour after the swim stress was analysed, yielding 6 samples for analysis of monoamines after exposure to the swim stress. Immediately after the sampling procedure the rat was disconnected from the sampling apparatus and placed in the airtight halothane enclosure until immobile. Cresyl violet (0.1%) was injected via the inlet of the microdialysis probe, the rat was placed back in the halothane enclosure for 7 minutes (recommended staining time 5-10 minutes) to allow the cresyl violet to stain the area of the brain adjacent the tip of the microdialysis probe. Hereafter the rat was decapitated and the brain was removed in order to determine the probe position.

The microdialysis probe has a 4 mm membrane working length and a 6 kDa cut off (acquired from TSE systems). Isotonic aCSF was used as perfusion fluid in this study. The fluid was kept at room temperature and a flow rate of 1.0 µl per minute was employed yielding 20 µl per sample in each 20 minute collection interval. Each sample was removed from the microfraction collector and immediately placed in an amber vial and placed in the auto-injector of the HPLC for analysis. Relative recovery was not determined in this qualitative study since the aim was to compare the results obtained in FSL rats treated with either fluoxetine or saline or in FSL rats exposed to either a 0 minute or a 10 minute acute swim stress. The concentrations of the various salts in the aCSF were (Wegener *et al*., 2000):

NaCl: 145 mM

KCl: 3.0 mM

$CaCl_2$: 1.2 mM

$MgCl_2$: 1.0 mM

Apparatus:

The syringe pump used: MAB 40 Dual Channel Microdialysis Pump (Microbiotech/Sweden)

Refrigerated microfraction collector: the 820 Microsampler (by Univentor)

Kopf® stereotaxic frame

A.1.6.5 Verification of Anatomical Probe Location

It is very important to ensure that the microdialysis probe has been placed in the correct anatomical position. Several methods have been developed using conventional tissue staining techniques. These methods are time consuming and are therefore impossible to do prior to the analysis of the samples which results in costly procedures being done to obtain results from samples which may not be useful in the study (Bert *et al*., 2004). An alternative technique developed by Bert and colleagues (2004) was used in this study because it is accurate, faster, and less wasteful than conventional staining techniques. It allows for the identification and exclusion of unusable samples prior to the initiation of the chosen chemical analysis procedure. Computer software is used to superimpose a digital photograph of a coronal section of the harvested brain (of the experimental animal) onto a digital image of an appropriate stereotaxic atlas representation of a coronal section of the animal brain. The track of the dialysis probe is visible and is used to verify the correct position of the probe against the atlas (Bert *et al*., 2004). This technique was combined with a conventional staining procedure in order to enhance the ease and accuracy with which the investigator could determine the probe position as indicated by the blue oval in Figure A-4 (see A.1.6.5.1 for a description of the staining procedure).

Immediately after removal the brain was placed in saline stored in a conventional fridge. Ice was added to the saline; the brain was left in this environment for 3 minutes. The brain was then transferred to a pre-chilled Kopf® PA 001 brain blocker which was then placed in a freezer (-20°C) and left overnight to freeze. This brain blocker was developed for use with the stereotaxic atlas of the rat brain developed by Paxinos, and Watson (Watson & Paxinos, 1989). On the next day successive sections were made with a single sided razor blade in the brain blocker until the stain could be identified. See Figure A-4 for an example.

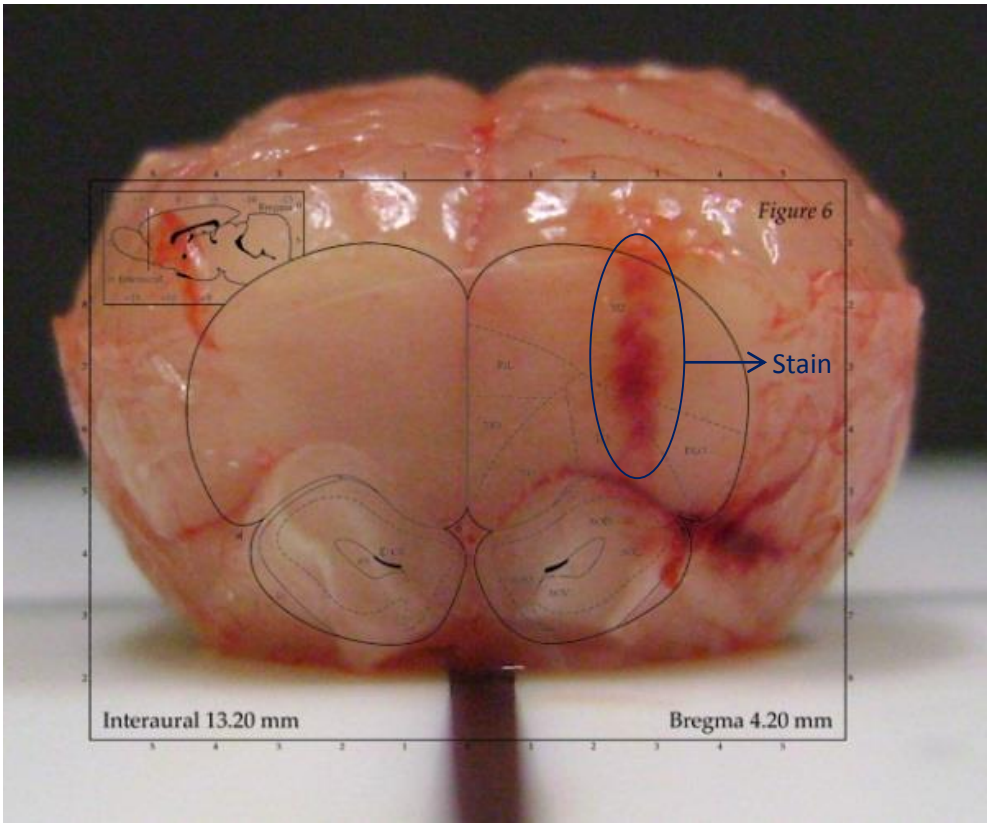

Figure A-4: Verification of probe position by means of cresyl voilet staining of the probe tract

A.1.6.5.1 Cresyl violet stain preparation

Cresyl violet acetate obtained from Sigma-Aldrich (CAS no: 10510-54-0, molecular formula: $C_{18}H_{15}N_3O_3$) was used. It was prepared by adding 0.1g of cresyl violet acetate to 100ml distilled water. Just prior to use 10 drops of glacial acetic acid was added and the solution was filtered (IHC World, 2014).

A.1.6.6 Sample analysis

Some of the analytes were in concentrations below the limit of detection of the HPLC analyses of the monoamines. It was therefore decided that only the monoamines and metabolites which could consistently be identified in the rat prefrontal cortex (see Figure A-5 for an example of chromatogram of microdialysis data), would be determined. Those were:

Norepinephrine (NE)

Dopamine (DA) and its metabolite homovanillic acid (HVA)

Serotonin (5HT) and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA)

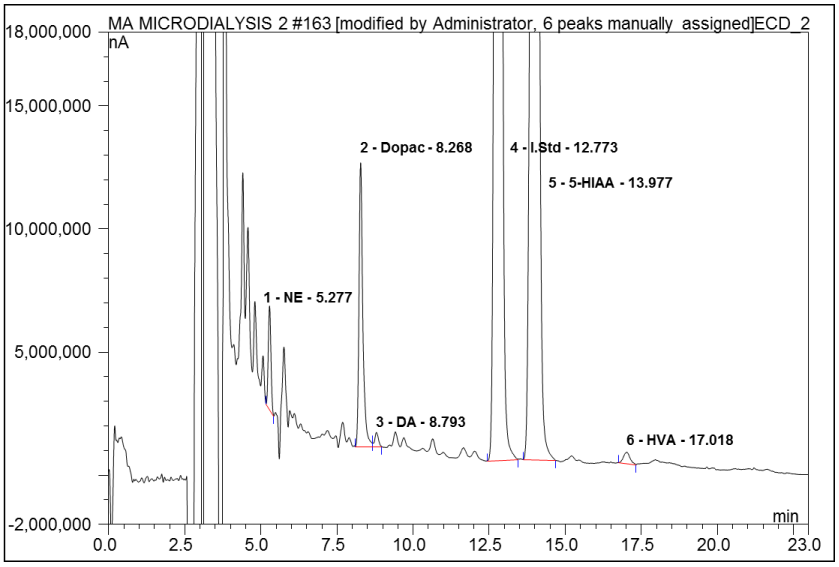

Figure A-5: Example of chromatogram from microdialysis experiments. NE= Norepinephrine, Dopac= 3,4-dihydoxyphenylacetic acid, DA= Dopamine, I.Std= Internal standard, 5-HIAA= 5-Hydroxyindole-3-acetic acid, HVA= Homovanillic acid.

This was the first time samples collected from in vivo microdialysis were assayed for monoamines in our laboratory. The validation procedures for the determination of monoamine concentrations in the small sample sizes of microdyalisate are discussed in A.1.6.6.5 (see figure A-6 for an example of 12.5 ng/ml HPLC standards).

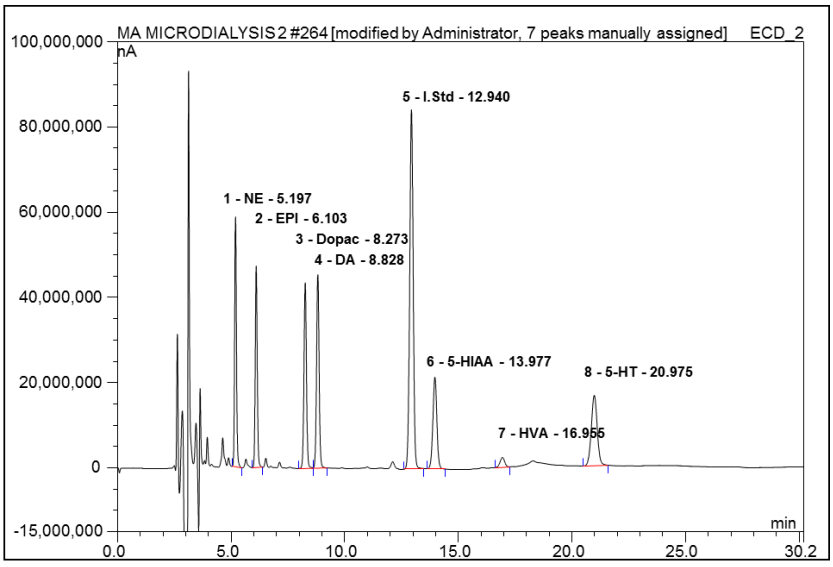

Figure A-6: 12.5 ng/ml HPLC standard. NE= Norepinephrine, EPI= Epinephrine, Dopac= 3,4-dihydoxyphenylacetic acid, DA= Dopamine, I.Std= Internal standard, 5-HIAA= 5-Hydroxyindole-3-acetic acid, HVA= Homovanillic acid, 5-HT= Serotonin.

A.1.6.6.1 HPLC electrochemical detection method

Table A-1: Chromatographic conditions.

Analytical instrument:	Agilent 1200 series HPLC equipped with and isocratic pump and autosampler
Electrochemical detector:	Coulochem III Electrochemical detector with a coulometric flow cell
Flow cell:	5014B Dionex Microdialysis Cell (Thermo Scientific)
Column	Kinetix 2.6µm C18, 100 Å, 150 x 4.6 mm, Phenomenex, Torrance, CA
Software:	Chromeleon® Chromatography Management System version 6.8.
Guard column	SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18, 4.0 x 3.0 mm, Phenomenex, Torrance, CA.
Mobile phase	ESA MDTM mobile phase
Flow rate	0.1 ml/min
Injection volume for instrument	20 µl
Coulometric electrochemical detector settings for instrument **E_1 – Test Electrode 1** **E_2 – Test Electrode 2** **E_{GC} – Guard Cell**	Settings for the coulometric flow cell: Model 5014B Dionex Microdialysis Cell (Thermo Scientific): Cell Potential Settings: E_1 = -150 mV, E_2 = +220mV, E_{GC} = +350mV, Range: 20 nA, Filter: 0.5 sec, Offset: 0%, Signal output: 1.0 V.

A.1.6.6.2 Mobile phase:

The ESA MDTM mobile phase was used (Liang *et al.*, 2008) which consists of:

75mM sodium dehydrogenate phosphate (monohydrate)

1.7mM 1-octanesulfonic acid (sodium salt)

100 ml/l triethylamine

25 µM EDTA

10% acetonitrile

The pH was adjusted to 3.00 with phosphoric acid.

A.1.6.6.3 Reagents

A.1.6.6.3.1 Solution A

Contents:

0.5 mM sodium metabisulphite (E Merck, Midrand).

0.3 mM ethylenediaminetetra-acetic acid disodium salt (Na_2EDTA) (E Merck, Midrand).

0.25 M perchloric acid ($HClO_4$) 60% solution (E Merck, Midrand).

Preparation:

0.047525 g sodium metabisulphite and 0.055836 g Na_2EDTA was weighed and dissolved in 400 ml of distilled water.

5.435 ml of perchloric acid was added to the above solution and topped up to 500 ml with distilled water.

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

A.1.6.6.3.2 Norepinephrine (NE) standard solution

L-norepinephrine hydrochloride = 205.6407 MW, norepinephrine = 169.1798 MW (82.27%).

1.22 mg of l-norepinephrine hydrochloride was weighed and dissolve in solution A (82.27% or 1mg of the 1.22 mg of powder constituted norepinephrine).

A.1.6.6.3.3 5-HIAA standard solution

5-HIAA (191.19 MW): 1 mg was weighed and dissolved in solution A.

A.1.6.6.3.4 Dopamine standard solution

3-Hydroxythyramine hydrochloride = 189.64 MW; Dopamine = 153.18 MW (80.77%).

1.24 mg of 3-Hydroxythyramine was weighed and dissolve in solution A (80.77% or 1mg of the 1.24 mg of powder constituted dopamine.

A.1.6.6.3.3 Homovanillic acid standard solution

Homovanillic acid (182.18 MW): 1 mg of the powder was weighed and dissolved in solution A.

All the above analytes were dissolved together in 10 ml of solution A in a 10 ml amber volumetric flask. This solution served as the standard stock solution and all the analyte concentrations were 100 µg/ml. From this solution a range of concentrations can be prepared to create a standard curve. All of the working standards were prepared from this solution as seen in table A-2.

Table A-2: Preparation of standard solutions

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

A.1.6.6.3.4 Preparation of internal standard (I.Std)

Isoproterenol hydrochloride (Isoprenaline)

1 mg of dl-isoproterenol hydrochloride was weighed and dissolved in 10 ml of solution A. This solution was used as the stock solution for the internal standard. Solution A was added to 30 µl of the stock solution up to total volume of 2 ml. This final solution was used as the working internal standard solution (concentration= 1500 ng/ml).

A.1.6.6.4 Microdialysis sample preparation

Samples were collected in 100 µl HPLC glass inserts (Agilent, Germany) placed in the refrigerated microfraction collector. 5 µl of solution A was added to each vial before sampling was initiated. The inserts were placed into amber HPLC vials and then transferred to the autosampler. The instrument's software was programmed to inject 20 µl onto the HPLC column, and analysed NE, 5-HIAA, DA, and HVA at testing electrode 2 with potential set at +220 mV. Results were expressed as ng/ml (nanograms per millilitre) and converted to nM using the following formula: (concentration (in ng/ml) x 1000)/molecular weight of the specific anylate).

A.1.6.6.5 System Suitabillity (mini validation) of the HPLC method used

The following parameter measurements were analysed to prove system suitability of the HPLC method used for the microdialysis study.

A.1.6.6.5.1 Linearity

The relationship between an instrument's response and the known concentrations of the analyte of interest is expressed in a linearity curve. A linearity curve should be created for each analyte in a sample and each analyte should have its own linearity curve. Four concentrations (standards) of each analyte were used to generate a linearity curve for the expected analytical concentration ranges. The standards for the linearity curve were prepared in a matrix close to the biological matrix of the endogenous compound, viz aCSF.

It is important that the linearity (standard) curve should have a linear regression value not less than $r^2 = 0.95$, as this curve will be used as a calibration curve to calculate the values of unknown concentrations in the test samples in the study (Visser, 2008).

The regression value for each analyte was as follows: Norepinephrine $r^2 = 0.9988$, 5-HIAA $r^2 = 0.9944$, Dopamine $r^2 = 0.9974$ and HVA $r^2 = 0.9936$ respectively.

A.1.6.6.5.2 Accuracy and precision

To validate accuracy and precision, samples of 3 concentrations (6.25, 12.5, and 25.0 ng/ml) were injected over 5 days to calculate the inter-day %RSD. The % RSD determined at each concentration level should be less than 15% (FDA, 2001).

Table A-3: %RSD of each analyte

Monoamine analyte	Concentration (ng/ml)	RSD (%)	*n- value*
Norepinephrine	6.25	6.29	5
	12.5	7.15	5
	25	2.41	5
5-HIAA	6.25	1.42	5
	12.5	9.47	5
	25	2.19	5
Dopamine	6.25	5.82	5
	12.5	9.39	5
	25	1.84	5
HVA	6.25	3.19	5
	12.5	8.71	4
	25	2.86	5

A.1.6.6.5.3 Lower limit of quantification (LLOQ) and lower limit of detection (LLOD)

The lower limit of quantification is the lowest concentration of the analyte of interest which can be quantitatively determined. The lower limit of detection is the lowest concentration of the analyte of interest that can be detected in a sample but not necessarily quantified under the stated analytical conditions. It is also defined as the lowest concentration that can be distinguished from the baseline and background noise with a certain degree of confidence.

The value for both the LLOQ and the LLOD for the method was 0.1 ng/ml for each analyte.

A.2 Results

A.2.1 Induced hypothermic response

Figure A-7 depicts the diffrences in 8-OH-DPAT induced hypothermic response between FSL and FRL rats.

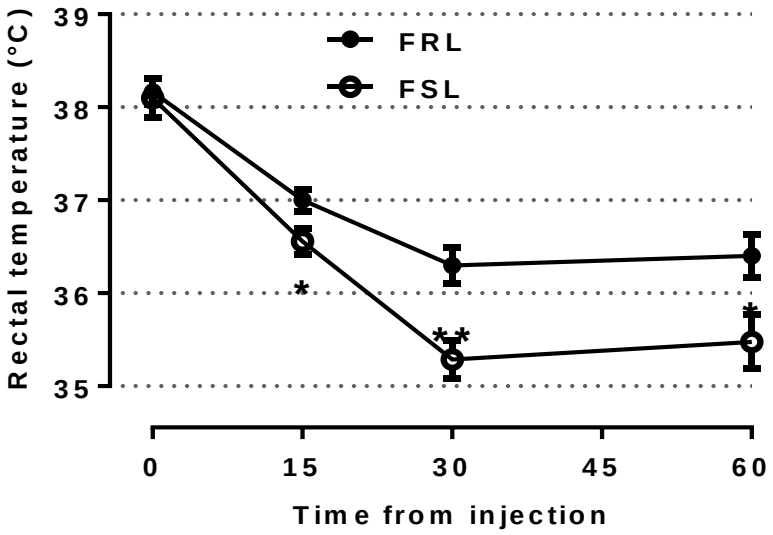

Figure A-7: Hypothermic response to s.c. 0.25mg/kg 8-OH-DPAT of FSL vs. FRL rats (n=9/strain). The data is presented as means and S.E.M.; two-tailed, unpaired Student-t test was performed with a value of $p<0.05$ taken as significant. Statistics: *$p<0.05$; **$p<0.01$.

Immediately after 8-OH-DPAT injection (0.25mg/kg s.c.) there was no significant difference between FSL (n=15) and FRL (n=15) rats. 8-OH-DPAT did provoke significant differences between FRL and FSL rats at 15 ($p= 0.0298$), 30 ($p= 0.0023$), and 60 ($p= 0.0238$) minutes after the injection (see Figure A-7).

A.2.2 Developmental effects of pre-pubertal fluoxetine administration on anxiety-like and depressive-like behaviours

A.2.2.1 Anxiety-like behaviour

Figure A-8 depicts the anxiety-like behaviour of FSL rats as measured in the OFT.

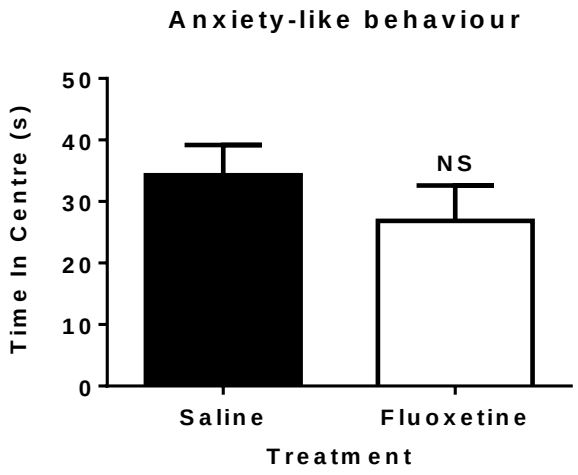

Figure A-8: Anxiety-like behaviour data of of FSL rats after treatment with saline or fluoxetine. Data points represent the mean and S.E.M., n= 15 rats per group (Student's t-test). NS= not significant.

It can be seen in Figure A-8 that saline-and fluoxetine-treated rats did not show any significant differences in anxiety-like behaviours in the OFT (34.28 ± 4.921vs 26.83 ± 5.762).

A.2.2.2 Depressive-like behaviour in the FST

Figure A-9 depicts the swimming and climbing behaviours of FSL rats as measured in the FST.

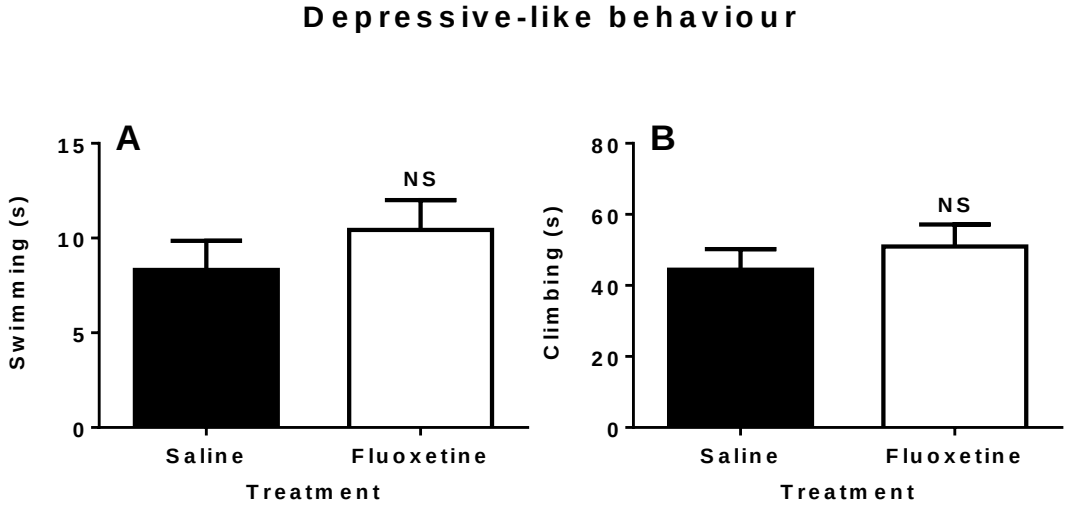

Figure A-9: (A) swimming behaviour and (B) climbing behaviour data of of FSL rats in the FST after treatment with saline or fluoxetine. Data points represent the mean and S.E.M., n= 15 rats per group (Student's t-test). NS= not significant

There were no statistically significant difference in swimming behaviour between saline-treated rats and fluoxetine-treated rats (8.313 ± 1.538 vs.10.43 ± 1.586) (Figure A9-A). There were no statistically significant differences between saline-treated rats and fluoxetine-treated rats in climbing behaviour (44.45 ± 5.815 vs. 50.99 ± 6.193) (Figure A-9B).

A.2.2.3 Stress response

A.2.2.3.1 Phase 1A: Corticosterone stress response

See article presented in chapter 3.

A.2.2.3.2 Phase 1B: Monoaminergic stress response

Figure A-10 depicts the indicated monoamine concentrations in the prefrontal cortex of stressed and unstressed FSL rats, before, during and after the 10 minute forced swim stressor (unstressed rats were not exposed to a 10 minute forced swim stressor).

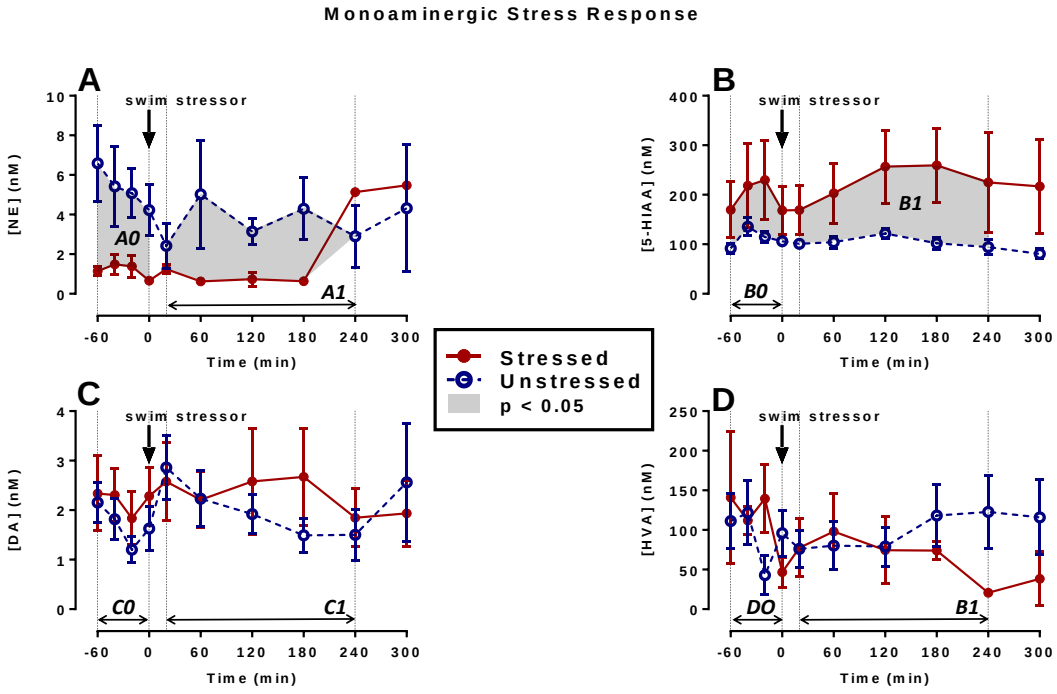

Figure A-10: Monoamine concentration of unstressed vs. stressed rats. Unstressed rats were not exposed to a 10 minute forced swim stressor. Data represented as means and S.E.M. n=5 unstressed group, n=4 stressed group. [NE]= concentration *l*-norepinephrine, [5-HIAA]= concentration 5-hydroxyindole-3-acetic acid, [DA]= concentration dopamine, [HVA]= concentration homovanillic acid.

In Figure A-10 two-way ANOVA analyses with group factor and time factor as repeated measure were performed on the data of the indicated monoamine levels (stressed and

unstressed) between -60 to 0 min before the swim (compare shaded area A0, A1, B0, B1) and again between 20 and 240 min after the swim (compare shaded area A1).

In Figure A-10A, area A0 there was no significant interaction between stressed and unstressed animals ($F [2, 10] = 0.48$, $p = 0.63$). There was a significant group effect ($F [1, 10] = 17.56$, $p = 0.0019$), indicating that the NE levels of unstressed (5.39 ± 1.30 nM) were significantly higher than stressed (0.52 ± 1.35 nM) animals In Figure A-10A, area A1 there was no significant interaction between stressed and unstressed animals ($F [4, 14] = 1.01$, $p = 0.4375$). There was a significant group effect ($F [1, 14] = 5.41$, $p = 0.0355$), indicating that NE levels in the prefrontal cortex were significantly higher in unstressed animals (4.46 ± 1.24) than in stressed animals (1.99 ± 1.36).

In Figure A-10B, area B0 there was no significant interaction between stressed and unstressed animals ($F [2, 16] = 0.18$, $p = 0.8355$). There was a significant group effect ($F [1, 16] = 4.53$, $p = 0.0492$), indicating that the 5-HIAA levels of stressed (208.20 ± 39.94 nM) were significantly higher than unstressed (115.10 ± 37.07 nM) animals In Figure A-10B, area B1 there was no significant interaction between saline- and fluoxetine-treated animals ($F [4, 30] = 0.60$, $p = 0.0.6667$). There was a significant group effect ($F [1, 30] = 9.59$, $p = 0.0042$), indicating that 5-HIAA levels in the prefrontal cortex were significantly higher in stressed animals (231.85 ± 34.83) than in unstressed animals (117.28 ± 32.12).

In Figure A-10C, area C0 there was no significant interaction between stressed and unstressed animals ($F [2, 16] = 0.09$, $p = 0.9167$). There was no significant group effect ($F [1, 16] = 0.65$, $p = 0.4306$), indicating that DA levels in the prefrontal cortex were not significantly higher in unstressed animals (1.71 ± 0.35 nM) than in stressed animals (2.09 ± 0.38 nM) before the swim stressor. In Figure A-10C, area C1 there was no significant interaction between stressed and unstressed animals ($F [4, 30] = 0.48$, $p = 0.7466$). There was no significant group effect ($F [1, 30] = 0.70$, $p = 0.4078$), indicating that DA levels in the prefrontal cortex were not significantly higher in unstressed (2.00 ± 0.40 nM) than in stressed animals (2.37 ± 0.44 nM) after the swim stressor.

In Figure A-10D, area D0 there was no significant interaction between stressed and unstressed animals ($F [2, 13] = 1.47$, $p = 0.2667$). There was a significant group effect ($F [1, 13] = 11.46$, $p = 0.0049$), indicating that HVA levels in the prefrontal cortex were significantly higher in stressed animals (214.87 ± 43.32 nM) than unstressed animals (86.52 ± 37.95 nM). In Figure A-10D, area D1 there was no significant interaction between saline- and fluoxetine-treated animals ($F [4, 20] = 0.69$, $p = 0.6057$). There was no significant group effect ($F [1, 20] = 1.81$, $p = 0.1931$), indicating that HVA levels in the prefrontal cortex were not significantly

higher in unstressed animals (97.20 ± 21.85 nM) than in stressed animals (61.95 ± 28.40 nM) after the swim stressor.

A.2.2.3.3 Developmental effects of pre-pubertal fluoxetine administration on monoaminergic stress response

See article presented in chapter 3.

A.3 Discussion

A.3.1 Anxiety-like behaviour in the OFT

No significant changes were seen in anxiety-like behaviour in FSL rats treated with pre-pubertal fluoxetine when compared to saline controls. These findings are in keeping with the fact that increased anxiety-like behaviour is not a feature typically observed in FSL rats (Neumann et al., 2011; Overstreet et al., 2005). The FSL rat has therefore been considered by some to be a selective animal model for depression (Overstreet, 1993).

A.3.2 Swimming and climbing behaviour in the FST

No significant changes were seen in swimming and climbing behaviours in the FST in FSL rats treated with pre-pubertal fluoxetine when compared to saline controls. Increases in one or both of these behaviours may have been masked due to decreased locomotor activity discussed in chapter 3.

A.3.3 Induced hypothermic response

Prior to the commencement of the corticosterone, microdialysis, and behavioural studies 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, was used to elicit a hypothermic response in both FSL and FRL rats. As seen in Figure A-6 the FSL rats displayed an exaggerated hypothermic response to 8-OH-DPAT injection at 15, 30, and 60 minutes.

A distinct exaggerated hypothermic response to 8-OH-DPAT injection due to muscarinic and serotonergic supersensitivity in FSL rats compared to FRL rats has been shown in other studies (Overstreet *et al*., 1994; Van Zyl, 2008). Overstreet and colleagues (2005) have made use of both cholinergic and serotonergic challenges in order to ensure the integrity of the FSL and FRL rat lines. This is necessary due to the fact that the animals in both strains are more than 90% inbred and genetic drift may occur between different breeding colonies in laboratories around the world (Overstreet *et al*., 2005).

A.3.4 Monoaminergic stress response

Phase 1B of the study was meant to support Phase 1A (corticosterone stress response, see article) and the developmental changes of the monoaminergic stress response associated with pre-pubertal fluoxetine injections in male FSL rats. Due to unforeseen but necessary renovations to the adjacent building (which is connected to the building which houses the microdialysis laboratory) at North-West University the data generated in this study could not be reliably interpreted. The renovations comprised of drilling into the foundations of the building causing considerable noise and vibration. I therefore hypothesise that stress caused to the stress sensitive FSL rats is responsible for the contradictory data. Support for this hypothesis is provided by the mean norepinephrine concentrations showing that the unstressed group released significantly more NE for the duration of the experiment than the stressed group during the -60 to 0 minute and the 20 to 240 minute sampling intervals. This occurred despite the absence of any swim stress in the unstressed group. This should be kept in mind when looking at the data presented for the analytes below.

A.3.4.1 NE stress response

Considering the significantly increased plasma corticosterone levels stimulated by the swim stress in stressed rats (see chapter 3 for corticosterone stress response findings) as well as the workings of the HPA-axis (discussed in 2.4.3) it was expected that NE concentrations would be elevated in the stressed group after the swim stress. The NE concentrations of the stressed group after the swim were increased relative to baseline concentrations of NE in this group before the swim which suggests that the swim stress successfully induced stress in this group of rats. The unstressed rats had a significantly higher baseline in terms of NE release in the prefrontal cortex prior to and after the swim stress. This suggests that the unstressed group was significantly stressed before the swim stress.

Acute tail pressure (30min) increased DA release in the medial prefrontal cortex in rats by 50% measured with microdialysis (Finlay *et al*., 1995). Smagin and colleagues (1995) showed that infusion of corticotropin releasing hormone into the locus coeruleus results in increased NE concentrations, measured with microdialysis, in the medial prefrontal cortex.

A.3.4.2 5-HIAA stress response

In this study the concentrations remained elevated from baseline for 2 hours after the rat was withdrawn from the stressor. The stressed group is shown to have elevated serotonin release and turnover at baseline conditions and after exposure to the swim stress. The

increases in 5-HIAA concentrations after the swim in the stress group is more marked in the stressed group than the unstressed group. It is of concern however that the stressed group had significantly increased mean 5-HIAA concentrations compared to unstressed rats before the swim stress.

Fujino and colleagues (2002) showed that 5-HT concentrations in the frontal cortex of mice increases by 240% after exposure to a 3 minute swim stress. Kirby and colleagues (1995) found no increase in 5-HT concentrations after a 30 minute swim stress. They also reported a decrease from pre-stress concentrations in 5-hydroxyindole-3-acetic acid (5-HIAA), the metabolite of 5-HT, of 45-60% after the swim stress in the striatum, ventral hippocampus, frontal cortex, amygdala, and lateral septum was significantly elevated in the stressed group prior to and after the swim stress. In contrast Reuter and Jacobs (1996) reported a significant increase in both 5-HT and 5-HIAA concentrations in response to a 30 min swim stress in Sprague-Dawley rat forebrain microdialysis samples.

A.3.4.3 DA stress response

No significant differences were seen in terms of DA concentrations between the rats in the unstressed and the stressed groups. The stressed group showed an increase in mean DA concentration after the swim stress. The unstressed group showed a mean DA concentration increase by approximately the same amount after the swim stress interval as the stressed group. This occurred despite the absence of the swim stress. The only difference between the two groups appears to be the baseline concentrations of DA which suggests that the increased DA concentrations seen in the stressed group is not due to the swim stress.

Finlay and colleagues (1995) showed an increase in DA concentrations of 54% in medial prefrontal cortex of rats after exposure to 30 minutes of acute tail pressure. Abercrombie and colleagues (1989) reported an increase of DA concentrations of 95% in the medial frontal cortex in response to an intermittent tail-shock stress.

A.3.4.4 HVA stress response

In the stressed group it would seem that the stressed rats showed a much higher mean DA turnover (as expressed by its end stage metabolite HVA) at baseline conditions than the unstressed group. After the swim stress the mean HVA concentrations decreased which suggests that the swim stress resulted in an attenuation of DA release and turnover in the prefrontal cortex. In the unstressed group there appears to be a gradual increase in DA turnover over the duration of the experiment. The HVA data also suggests that the stressed

rats were significantly more stressed than the unstressed rats prior to but not after the swim stress.

Significant increases were seen in HVA concentrations due to intermittent tail-shock stress (Abercrombie *et al*., 1989). Kaneyuki and colleagues (1991) showed that HVA concentrations in the medial PFC of psychologically stressed rats were increased when exposed for 30 minutes to the emotional responses of rats exposed to foot-shock stress.

Addendum B: 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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