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Brain concentrations and the neurochemical effects of passively administered fluoxetine in Flinders sensitive line rat offspring

by

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Research report

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DECLARATION

I, Stephan Steyn, hereby declare that all experimental work, planning, literature research, data analyses, interpretation, and writing this dissertation was done by myself. This project was funded by research funds awarded to me by the North-West University and approved by the necessary Ethics and Research committees. All neurochemical analyses were performed by me, with the assistance of Doctor Francois Viljoen of the North-West University, Pharmacen research entity. All statistical analyses were also performed by myself.

I also declare that:

- I am aware that plagiarism is wrong, and have therefore subjected this report to a similarity comparison, of which the summary report is available in Addendum B.
- I confirm that the work submitted for assessment for the degree mentioned on the cover page (Master of Science in Clinical Pharmacy) is my own unaided work (except for the above-mentioned assistance).
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work, or that I have failed to acknowledge the source of the ideas or words in my writing.

Stephanus F Steyn

ABSTRACT

Background: Globally, 36 % of women who have recently given birth, experience symptoms of depression and anxiety. Effective antidepressant treatments are limited, with fluoxetine being a popular treatment option. Fluoxetine is expressed in the breast milk, yet it is unclear to what extent fluoxetine, or its active metabolite, norfluoxetine, reaches the brain of the developing child and what the effects of such exposure on the related neurobiological processes would be. Due to ethical considerations and practical restrictions, clinical investigations into the neurodevelopmental effects of passively administered antidepressants (via the breast milk) are problematic. Therefore, pre-clinical investigations into this topic are not only important but clinically relevant. **Aims & objectives:** We aimed to quantify the concentration of passively administered, i.e., via the breast milk during nursing, fluoxetine, and its active metabolite, norfluoxetine in the whole brains of exposed Flinders sensitive line (FSL) rats (an established rodent model of depression). We further aimed to establish if said exposure would associate with changes in whole-brain serotonergic function and redox status. **Methods:** Adult FSL dams received fluoxetine (10 mg/kg/day), or placebo for fourteen days, beginning on postpartum day 04. Offspring (*n* = 16 per exposure group; 1:1 male: female) were passively exposed to fluoxetine until postnatal day 18 and euthanized on postnatal day 22. Whole brain fluoxetine, norfluoxetine, serotonin, 5-hydroxyindoleacetic acid (5-HIAA), and reduced and oxidized glutathione (GSH and GSSG) concentrations were measured via liquid chromatography/mass spectrometry (LC-MS). **Results:** Fluoxetine, was undetectable in the brain of FSL offspring, while norfluoxetine concentrations, averaged 41.28 ± 6.47 ng/g. Neither serotonin, nor its metabolite (5-HIAA), was affected by passively administered fluoxetine in the juvenile brain. In terms of redox status, pups exposed to fluoxetine presented with a compromised antioxidant defence, as evinced by a lower GSH/GSSG ratio. **Discussion and conclusion:** Although fluoxetine and norfluoxetine concentrations have been measured in breast milk and infant plasma, to the best of our knowledge, it has not been quantified in the juvenile brain until now. Our results are in line with clinical findings, suggesting the infant norfluoxetine/fluoxetine ratio to be elevated, probably because of the prolonged half-life of norfluoxetine. Although only norfluoxetine was detected, this did not influence the central serotonin concentrations of offspring. However, it associated with increased oxidative stress, of which the pathophysiological significance remains to be established. Taken together, our findings confirm that passively administered fluoxetine does reach the infant brain in the form of norfluoxetine and may manipulate processes of oxidative stress regulation. Further studies into the long-term bio-behavioural effects are however needed to effectively inform breast feeding mothers on the safety of antidepressant-use during the postpartum period.

Keywords

Breast milk; Flinders resistant line (FRL); Flinders sensitive line (FSL); Norfluoxetine; Oxidative stress

Highlights

- Passively administered fluoxetine results in detectable concentrations of norfluoxetine, but not fluoxetine, in whole brains of pre-pubertal male and female FSL rats.
- Serotonergic metabolism and concentrations are unaffected by chronic passively administered fluoxetine.
- Pre-pubertal male and female FSL rats have reduced whole antioxidant defences (GSH/GSSG), compared to age matched FRL controls.
- This GSH/GSSG imbalance is further reduced by chronic passively administered fluoxetine.

Limitations

- The body weight of the pups was not available to validate whether brain weights were indeed comparable across the different experimental groups.
- Because only GSH and GSSG concentrations were measured, concluding on whether the observed redox state differences are indeed detrimental to juvenile development, remains to be confirmed.
- That no behavioural analyses could be performed, further limits the interpretation of the nature of these results.

List of abbreviations

5-HIAA: 5-hydroxyindoleacetic acid; **5-HT:** 5-hydroxytryptamine (serotonin); **8-OHdG:** 8-hydroxy-2'-deoxyguanosine; **ACN:** Acetonitrile; **ATP:** Adenosine triphosphate; **BDNF:** Brain-derived neurotrophic factor; **CRH:** Corticotropin-releasing hormone; **DNA:** Deoxyribonucleic acid; **DSM-V:** Diagnostic and statistical manual of mental disorders, 5th edition; d_{unb}**:** Unbiased Cohen's *d*-value; **FLX:** Fluoxetine; **FRL:** Flinders resistant line; **FSL:** Flinders sensitive line; **GABA:** Gamma aminobutyric acid; **GSH:** Glutathione (reduced); **GSSG:** Glutathione disulphide (oxidized); **HPA:** Hypothalamic-pituitary-adrenal; **nFLX:** Norfluoxetine; **NWU:** North-West University; **PND:** Postnatal day; **PPD:** Postpartum day; **SSRI:** Selective serotonin re-uptake inhibitor; **Wits:** University of the Witwatersrand.

Dedicated to my parents, wife and two hooligans

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CHAPTER 1: INTRODUCTION

1.1 Dissertation layout

The study rationale, methodology, findings, and discussion thereof are presented across five chapters. The problem statement, research questions, and working hypothesis for the project are presented in Chapter 1, together with the specific aims and layout of the study. In Chapter 2, a review of the available and relevant literature is given to serve as background for the results of this project and give context for the interpretation thereof. Briefly, Chapter 2 reviews the prevalence of postpartum depression, available treatment options and the possible neurodevelopmental effects of these interventions on the developing brain. This chapter then concludes with a review of available literature, related to the validity of the Flinders sensitive line rat (strain used in the current study) for this research report. Next, the relevant materials and experimental methods used in the current study are described in Chapter 3. Chapter 4 contains the results and findings, together with a detailed description of the statistical methods used, and discussion of the findings against the background of relevant literature. These findings are then summarised in Chapter 5, specifically in response to the various research questions listed in Chapter 1. Based on this discussion, this chapter concludes with a general answer to the overarching research question, posed in Chapter 1. The conclusion is finally used to highlight specific study limitations and direct future research into this topic.

Two addenda are also included in this report to supplement the main body, described above. These include ethical approval certificates from the two relevant Institutions (*Addendum A*), and the Turnitin summary report (*Addendum B*).

To ease reading, a comprehensive reference list is presented at the end of the dissertation (as opposed to after each Chapter).

This dissertation is written in UK English.

1.2 Background and problem statement

The first year postpartum is a particularly sensitive period in a women's life, posing significant health risks to both mother and infant (Vernon *et al.*, 2010). Healthcare-associated factors that can be adversely affected during this period include general medical care and costs, breastfeeding, family function and even early brain development in the infant due to suboptimal maternal care (Rafferty *et al.*, 2019). Symptoms of maternal psychological distress can include depression, anxiety, and stress during the postpartum period (Trujillo *et al.*, 2018), which can all contribute to the worsening of the aforementioned factors but also increase the risk to develop other neuropsychiatric disorders, such as obsessive-compulsive disorder (Zambaldi *et al.*, 2009), post-traumatic stress disorder (Grekin & O'Hara, 2014), and anxiety (Tietz *et al.*, 2014). Worldwide, 10 % of pregnant women and 13 % of women who have recently given birth, experience a mental disorder (Centers for disease control and prevention, 2023; Gavin *et al.*, 2005), regardless of familial history of postpartum depression (Sullivan *et al.*, 2000). In South Africa, as much as 37 % of women struggle with depression during the peripartum period – the second highest out of 56 countries (Hahn-Holbrook *et al.*, 2018). Despite early reports indicating postpartum depression prevalence to have increased seven-fold between 2000 and 2015 (Centers for disease control and prevention, 2023), more recent reports point to an even worse prevalence, exacerbated by the recent global Covid-19 pandemic. In fact, the prevalence of maternal psychological stress has increased to such an extent, that as much as 36 % of mothers of children younger than 18 months (Cameron *et al.*, 2020) reported depression and anxiety symptoms – an overall increase of 8 % during the pandemic (from 26 to 34 %) (Wu *et al.*, 2020). Yet, despite these numbers and that universal screening for maternal depression during the perinatal period is recommended by professional bodies, as little as 40 % of women who experience depression during this period, seek medical help (Rafferty *et al.*, 2019).

When left untreated, postpartum depression may lead to suicide, which severely and adversely affects the physical and bio-behavioural development of the new-born child (Frieder *et al.*, 2019). Moreover, untreated, and even undiagnosed postpartum depression can lead to infanticide, which in turn can have devastating effects on the overall wellbeing of the family. In fact, depression is one of the most commonly diagnosed mental disorders in school-aged children (Centers for disease control and prevention, 2020). It is the fourth leading cause of global illness and disability among adolescents (15-19 years of age) and the fifteenth in those younger than 14 years (World Health Organization, 2022). In the United States, almost two million children (aged 3-17 years) have been diagnosed with depression (Centers for disease control and prevention, 2020; Ghandour *et al.*, 2019), with data indicating that 17 % of children (2-8 years), 5 % of 12-year-olds and 17 % of 17-year-olds have at least experienced a major depressive episode in the year before (Centers for disease control and prevention, 2020; Cree *et al.*, 2018; Selph & McDonagh, 2019). For the South African context, available data suggests that between 13 and 33 % of adolescents struggle with depressive symptoms (Barhafumwa *et al.*, 2016; Nduna *et al.*, 2013). In 2016, an estimated 62 000 adolescents died as a result of self-harm (World Health Organization, 2022), making it the third leading global cause of death in this age group, with the majority (> 90 %) of cases reported in low- or middle-income countries (World Health Organization, 2022).

Regardless, the only currently FDA[1]-approved pharmacological treatment option for mild to severe postpartum depression, is brexanolone (a gamma-aminobutyric acid A receptor positive allosteric modulator) (Payne, 2021). However, it is only available as a continuous intravenous infusion, and may not yet be widely available in all countries, consequently limiting its general use due to practical and financial reasons. Alternatively, the selective reuptake inhibitor (SSRI), fluoxetine, is generally used as a first-line option alternative (Frieder *et al.*, 2019). Fluoxetine crosses the placenta and is expressed in breast milk, which could potentially pose a health risk to the infant (Frieder *et al.*, 2019; Gao *et al.*, 2018). This often results in pregnant and breastfeeding women being sceptical to use antidepressants during the peripartum (including early postpartum) period (Battle *et al.*, 2013; Prady *et al.*, 2016). Importantly, refusal of (pharmacological) antidepressant treatment can negatively influence the development of a child, as postpartum depression can lead to maternal neglect (Dietz *et al.*, 2008), which could set off a range of adverse, and as mentioned, even fatal consequences.

Fluoxetine increases serotonin levels by inhibiting the serotonin transporter, thereby preventing the re-uptake of serotonin into the neuron (DeBattista, 2021). Despite functional polymorphisms affecting the activity of the transporter, SSRIs, such as fluoxetine, inhibit approximately 80 % of the transporter at therapeutic doses (DeBattista, 2021), thereby explaining the clinical popularity of this class of antidepressants. The active metabolite of fluoxetine, norfluoxetine, has an elimination half-life three times greater than that of the parent compound, resulting in prolonged pharmacodynamic effects, even after cessation of treatment (DeBattista, 2021). In fact, monoamine oxidase inhibiting drugs must be avoided for at least four weeks following treatment cessation to avoid serotonin syndrome (DeBattista, 2021). Secondary to the serotonergic-enhancing effects, fluoxetine also affects mitochondrial function. This is of note as mitochondrial (dys)function has recently gained more attention as a promising and novel target to treat depression (Allen *et al.*, 2018; Sharma & Akundi, 2019; Wu *et al.*, 2019). Briefly,

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[1] *United States Food and Drug Administration*

mitochondria provides 80 % of cellular needs in the form of adenosine triphosphate (ATP) (Papa *et al.*, 2012) that is necessary for neurotransmission (calcium-regulating properties), neuronal membrane potential maintenance (Cotman & Berchtold, 2002; Mattson *et al.*, 2008; Sheng & Cai, 2012), and neurogenesis (processes that affect neuroplasticity), of which the latter is strongly associated with mitochondria number (Chen *et al.*, 2018). Despite accounting for just 2 % of a person's weight, the human brain requires 20 % of our total glucose and oxygen intake (Manji *et al.*, 2012; Pei & Wallace, 2018; Rolfe & Brown, 1997), with the majority of energy (80 to 90 % of the total brain demand) required for neuronal function (Yu *et al.*, 2018). More specifically, energy is produced by oxidative phosphorylation within the mitochondrial electron transport chain, which, amongst others, produces reactive oxygen species as by-product (Adam-Vizi, 2005). In healthy mitochondria, this reactive oxygen species production is counterbalanced by mitochondrial-produced antioxidant defences (Tse *et al.*, 2016; Turrens, 2003), thereby preventing cellular damage and/or dysfunctional processes. Conversely, when mitochondria function sub-optimally, reactive oxygen species production is increased (Federico *et al.*, 2012; Klinedinst & Regenold, 2015). Without the necessary increase in antioxidant defences, an imbalanced redox status develops, that can lead to cellular damage and apoptosis (Federico *et al.*, 2012). Because increased oxidative stress is associated with various psychological conditions, including depression and anxiety (Lopresti *et al.*, 2014; Miller *et al.*, 2009), it is worth noting that mitochondrial dysfunction has also been linked to several underlying causes of or contributing factors to psychiatric conditions, such as impaired monoaminergic neurotransmission, reduced neuroplasticity, and increased neuro-inflammation (Klinedinst & Regenold, 2015; Mattson *et al.*, 2008; Sharma & Akundi, 2019). It is therefore significant that when reviewing the bio-energetic effects of fluoxetine, both positive and negative effects are observed and is reviewed by Emmerzaal and colleagues (2021). This is of particular interest when considered during the energy dependant neurodevelopmental processes of early-life development (i.e., early postpartum period).

With the above-mentioned considered, together with the fact that fluoxetine and norfluoxetine are expressed in breast milk, and the prolonged effect of the active metabolite, it is understandable that women may be hesitant to start or continue fluoxetine treatment whilst breastfeeding. Clinical data regarding the neurochemical effects of passively administered fluoxetine (i.e., via breast milk) in offspring are largely limited, because of the practical and ethical implications. The knowledge gained from pre-clinical research, is therefore invaluable. Available data suggests mixed results in terms of the effects of passively administered fluoxetine in offspring rodents, specifically during early life development. Studies that did in fact, investigate the effects of fluoxetine delivered via the breast milk in rodent offspring, found that increased anxiety-like behaviour, impaired hypothalamic-pituitary-adrenal (HPA) axis' negative feedback mechanism, and altered hippocampal immature neurons in a sex dependent manner during adulthood (Gobinath *et al.*, 2016). Furthermore, postpartum fluoxetine also decreased hippocampal markers of neuroplasticity (Boulle *et al.*, 2016), and prevented exercise-induced (Gobinath *et al.*, 2018) neurogenesis in adult offspring. In contrast, Zaccarelli-Magalhães and colleagues (2020) recently reported that although postpartum fluoxetine altered maternal behaviour, it had no effect on juvenile behaviour in the offspring born to these dams. Because the serotonergic system is still developing during the early postpartum period, it is also worth noting that passively administered fluoxetine influences sexual behaviour in adult offspring, again in a sex-dependant manner (Rayen *et al.*, 2013, 2014). Finally, an early clinical study, investigating the absolute concentration of fluoxetine in breast milk, found that the predicted maternal weight-adjusted daily dose of the infant ranges from 2 to 4 % at two weeks and two months of age, respectively (Heikkinen *et al.*, 2003). This was further quantified by Taddio and colleagues (1996), who reported a weight-adjusted dose of 0.17 ± 0.09 mg fluoxetine (equivalent to 10.8 ± 2.2 % of the maternal dose) to be administered to the new-born. However, the exact concentration of this passively administered fluoxetine to reach the developing brain, remains unknown.

As mentioned, the ethical considerations and implications for a clinical study during early-life development is an obvious and serious limitation, making pre-clinical investigations into this topic more important and relevant. To this end, the Flinders sensitive line (FSL) rat is an established, accepted and validated genetic rodent model of depression (Overstreet, 1993; Overstreet & Wegener, 2013), displaying behaviour and neurochemical constructs akin to the clinical condition. That these discrepancies are further successfully reversed by pharmacological (Overstreet & Wegener, 2013) and non-pharmacological antidepressant interventions (Steyn *et al.*, 2018; Steyn *et al.*, 2020), make it a translatable model to investigate the neurodevelopmental effects of passively administered fluoxetine in pre-pubertal offspring.

1.3 Research question

With the above-mentioned in mind, pre-clinical investigation into the neurochemical effects of passively administered fluoxetine on the developing brain is required. Moreover, by quantifying the amount of fluoxetine that reaches the developing brain may be of specific importance, specifically in a rodent model of depression (i.e., the FSL rat) with reliable clinical translatability. The current study therefore sets out to answer how much of passively administered fluoxetine (if any) actually reaches the juvenile brain, and what the effects thereof are on serotonin levels and redox state markers?

1.4 Study aims and objectives

To accurately answer this research question, various measurable study aims and objectives were set out. Firstly, we aimed to measure fluoxetine (and norfluoxetine) concentrations in the whole brains of 22-day old male and female FSL pups, born to dams treated daily with 10 mg/kg fluoxetine during the postpartum period. Secondary to this, and to inform on the neurodevelopmental consequences of passively administered fluoxetine, serotonin levels, and markers relating to redox state (refer to Table 1-1, below) were measured. Finally, and as context for these results, we also set out to establish baseline strain differences between FSL and FRL pups in terms of the mentioned neurochemical markers, thereby further validating the known depressive-like constructs of the Flinders strain.

Table 1-1: Study aims and objectives

The study aims and objectives summarized here, must be read with Figure 1-1.

Study aims	Objectives
1) Quantify the amount of fluoxetine that reaches the pre-pubertal brain in pups via passively (indirect) administration (i.e., via breast milk from treated lactating dams).	Measure the whole brain concentrations of fluoxetine and norfluoxetine in FSL pre-pubertal brain tissue (*Group 2 vs. 3*).
2) Confirm the neurochemical construct differences between the prepubertal FSL and FRL rats.	Measure markers of oxidative stress (GSH[1] and GSSG[2]) and serotonin levels in the pre-pubertal whole brain (*Group 1 vs. 2*).
3) Determine whether sex differences exist in the offspring of any of the above-mentioned objectives.	Compare the above-mentioned parameters between male and female pups via visual inspection and statistical analysis where obvious differences are observed.

1.5 Hypothesis

As a working hypothesis, we expect pre-pubertal FSL rats (regardless of sex) to have lower serotonin levels and increased markers of oxidative stress, relative to healthy FRL controls. Moreover, we hypothesize that passively administered fluoxetine will result in high norfluoxetine/fluoxetine values in the pre-pubertal brain and will reverse the mentioned serotonin and markers of oxidative stress to levels comparable with that of healthy FRL controls.

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[1] *reduced glutathione*

[2] *glutathione disulphide (oxidized)*

1.6 Study design and layout

The tissue used in the current study was ethically collected and preserved (AnimCare approval number: NWU-00434-21-A5) in a previous study, performed at the North-West University (Oosthuizen, 2022), where approval for analysing these samples in future studies (i.e., the current study) was given. For context, the study layout is summarized in Figure 1-1, below. Briefly, female FSL rats were administered fluoxetine (10 mg/kg/day) or distilled water (as control), subcutaneously, from postpartum day 04 (PPD04) until PPD18. Between PPD19 and 21, postpartum dams underwent a series of behavioural analyses, whereafter they (and the pups) were euthanized, via decapitation on PPD22 (postnatal day 22 for the pups). Whole brains of the pups were removed, snap frozen in liquid nitrogen and stored at -80 °C for neurochemical analyses (*see Chapter 3*). For the FRL group, postpartum FRL dams received vehicle control (i.e., distilled water) during the same intervention period, whereafter they were also euthanized on PND22. As summarized in Figure 1-1, below, a total of 48 offspring samples (1:1 male: female) were used (FRL, *n* = 16; FSL, *n* = 32) in the current study.

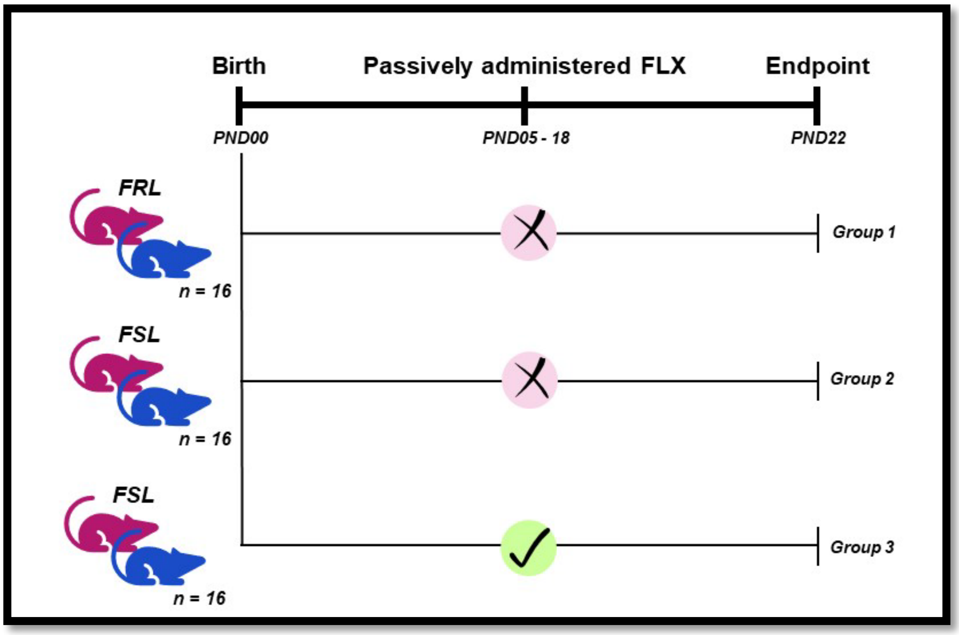

Figure 1-1: Graphical representation of study layout
Male (blue) and female (pink) Flinders sensitive and resistant line pups were either exposed to passively administered fluoxetine during early-life development (i.e., PND05 to 18), or not (Groups 1 and 2). Neurochemical analyses of control groups (Groups 1 and 2) will be compared to determine baseline differences of the juvenile brain. Because only depressed patients would be treated with FLX, only FSL rats born to FLX-treated dams will be included and compared to FSL controls. No statistical comparisons between Groups 1 and 3 will be performed. ***FLX:*** *Fluoxetine (10 mg/kg/day) administered to the dam.* ***FRL:*** *Flinders resistant line.* ***FSL:*** *Flinders sensitive line.* ***PND:*** *Postnatal day.*

1.7 Justification of group sizes

The results of an A priori test (*Table 1-2; Means: Difference between two independent means*) are presented below with a suggested 26 animals per group for the analyses at both PND21 confirm strain phenotype (FSL *vs.* FRL).

Table 1-2: Summary of power analyses performed, justifying the experimental group sizes

Input parameters		**Output parameters**	
A priori power analysis to determine group sizes			
Tails	Two	**Noncentrality parameter ς**	2.88
Effect size *d*	0.80 (*large*)	**Critical *t***	2.01
α err prob	0.05	**df**	50
Power (1 – β prob)	0.80	**Total sample size**	52 (*26 per group*)
Allocation ratio N2/N1	1	**Actual power**	0.81
Sensitivity analysis, supporting proposed group sizes			
Tails	Two	**Noncentrality parameter ς**	2.90
α err prob	0.05	**Critical *t***	2.04
Power (1 – β prob)	0.80	**df**	30
Sample size group 1	16	**Effect size *d***	1.02 (*large*)
Sample size group 2	16		

Importantly, recent studies have been able to identify statistical group differences between FSL and FRL rats, using between eight and twelves animals/group (Abildgaard *et al.*, 2011; Hesselberg *et al.*, 2016; Oberholzer *et al.*, 2018; Tillmann & Wegener, 2019), with others (Roets *et al.*, 2023; Whitney *et al.*, 2023b) also confirming the large effect strain difference. Consequently, a Sensitivity analysis (Table 1-2) supports the use of the proposed sixteen animals (8 male and 8 female) per group, with a less than 0.05 difference in required critical *t*-value. Therefore, the proposed sixteen animals per group should be sufficient to identify statistically significant group differences, as well as highlight sex differences, without neglecting research integrity. Importantly, all data sets were first visually inspected for any obvious sex differences, and where evident, separate statistical analyses were used to confirm these apparent differences.

1.8 Translatability of the findings and predicted impact

The findings of the current project would not only contribute to the greater neuroscience field but will also be of value to the clinical understanding of the long-term effects of passively administered fluoxetine on the developing brain. Moreover, and to the best of our knowledge, the concentration of passively administered fluoxetine that reaches the developing brain was quantified for the first time. Overall, if translated to the clinical setting, the findings of this project could inform healthcare providers about the safety of prescribing antidepressants to breastfeeding women and contribute to our limited understanding about the long-term effects of these medications in the developing brain.

1.9 Ethical considerations and approval

This study was approved by the AnimCare Ethics Committee of the North-West University (*approval number: NWU-00789-23-A5*; *Addendum A*), where the analyses were performed. Ethical approval was waived by the University of the Witwatersrand (*waiver nr: 25-04-2023-O; Addendum A*). All procedures complied with national legislation that pertains to experimental animal welfare (South African National Standard for the Care and Use of Animals for Scientific Purposes; SANS 10386:2008). The study also complied with the Animal Research: Reporting of *in Vivo* Experiments (ARRIVE) guidelines ensuring that all experimental data are reproducible, transparent, accurate comprehensive and logically ordered to promote well written manuscripts (du Sert *et al.*, 2020). To this end, the ARRIVE Essential 10 points are summarised in Table 1-3, below.

Table 1-3: ARRIVE Essential 10

ARRIVE Essential 10	**Summarized answer / Cross-reference to relevant section**
Study design	Section 1.6
Sample size	Sections 1.6 and 1.7
Inclusion and exclusion criteria	*Inclusion*: Brain tissue of male and female FSL and FRL pups. *Exclusion*: Any brain tissue with obvious damage.
Randomization	Because the samples were collected in a previously completed study, the samples are randomly stored (yet accurately labelled). Still, for the available samples, a free online randomization programme (*https://www.randomizer.org/*) was used to select the samples to be analysed.
Blinding	Not applicable as the samples were collected in a recently completed study (Oosthuizen, 2022).
Outcome measures	Section 1.6 and Chapter 3
Statistical methods	Sections 4.1
Experimental animals	Sections 1.6, 2.6 and 3.1
Experimental procedures	Chapter 3
Results	Chapter 4

CHAPTER 2: LITERATURE REVIEW

This chapter provides a review of the available literature on the topics relevant for the understanding and interpretation of the findings of the current study. To this end, the prevalence of postpartum depression, together with a brief overview of the aetiology of this mental illness is provided. The available treatment options to address to hypothesised aetiology of postpartum depression is then reviewed, with special attention given to fluoxetine. The possible neurodevelopmental effects of these interventions on the developing brain, is then provided, with the Chapter concluding with a brief justification of why the Flinders sensitive line (strain used in the current study) is suitable for the purpose of this research project.

2.1 Epidemiology of postpartum depression

Due to improved modern diagnostic tests and general increased social awareness, the prevalence of postpartum depression has increased over recent years (Dadi *et al.*, 2020). However, because of under reporting of diagnosed cases, the actual prevalence remains unknown. According to available data, the global prevalence of postpartum depression, regardless of age, giving birth for the first time (primiparous), or being a single mother, is estimated to be between 14 (Liu *et al.*, 2022) and 17 % (Shorey *et al.*, 2018). Although the prevalence of postpartum depression appears to peak at twelve months postpartum, this is statistically comparable to earlier time points (i.e., 0, 3 and 6 months) (Shorey *et al.*, 2018). Alarmingly, a significant increase in the prevalence of postpartum depression (specifically in low-income countries, such as Brazil, Peru, Thailand, Pakistan and South Africa (Gelaye *et al.*, 2016)) has been observed in more recent years, increasing from 18 % between 2010 and 2012 to 26 % in 2017 (Dadi *et al.*, 2020). This increase was further maintained by the recent global Covid-19 pandemic, with recent meta-analyses estimating between 27 and 34 % of pregnant and lactating women to now suffer from postpartum depression (Chen *et al.*, 2022; Safi-Keykaleh *et al.*, 2022).

This noteworthy impact of Covid-19 on the mental health of new mothers is not unique. In fact, generalized anxiety scores of healthcare workers was also found to be increased during the pandemic (Adibi *et al.*, 2021), thereby highlighting the impact of psychological stress on the development of mood disorders – specifically major depressive disorder. Psychological stressors (as those associated with Covid-19 lockdown regulations) is well-known to accelerate the occurrence of mental health conditions, such as postpartum depression, and is therefore considered a significant risk factor (Çankaya, 2020). Other noteworthy risk factors for postpartum depression, include gestational diabetes, giving birth to boys, having a history of depression (before or during pregnancy), exposure to traumatic events during or prior to pregnancy, and various socio-demographic factors, such as maternal age employment and marital status, and preterm delivery (Chen *et al.*, 2022; Doyle & Klein, 2020; Liu *et al.*, 2022).

As for the potential mechanisms of these risk factors, the hyperglycaemia and hormonal influences of gestational diabetes may adversely influence the thyroid and stress-axis (i.e., hypothalamic-pituitary-adrenal (HPA) axis) to such an extent, that the response to the sudden and unexpected stress burden of a chronic disease during pregnancy and the postpartum period, triggers depressive symptoms (Liu *et al.*, 2022; Zhang *et al.*, 2005). It must however be noted that in a recent pre-clinical study, Zhao and colleagues (2022) noted that although gestational diabetes induced depressive-like behaviour in postpartum Sprague-Dawley rats, serum corticosterone levels were actually decreased, thereby suggesting that the hyperglycaemic construct, actually inhibited the secretion of cortisol (or corticosterone in rodents) via negative feedback mechanisms. Either way, serotonin concentrations, and the metabolism thereof (tryptophan to serotonin), were decreased in these animals, aligning with the serotonergic dysfunction hypothesis of depression (Fakhoury, 2016; Kambeitz & Howes, 2015). This finding could explain why Clark and colleagues (2019) observed a unidirectional (as opposed to the assumed bi-directional) association of diabetes with depression, stating that depression precedes, but does not follow gestational diabetes. Nevertheless, that they further conclude trauma to increase the risk for both hyperglycaemia and depression (via prolonged HPA activation), links back to the mentioned sudden and unexpected stress impact of being diagnosed with a chronic disease, such as gestational diabetes or pre-eclampsia during pregnancy, of which the latter has been found to not only be a risk factor for depression but is also associated with more severe symptoms (Caropreso *et al.*, 2020).

Intriguingly, although giving birth to a boy has been highlighted as a risk factor for postpartum depression (Mori *et al.*, 2018; Pampaka *et al.*, 2019), the exact neuro-biological mechanism of this association remains to be established. It is therefore important to consider that Hall and Holden (2008) observed that giving birth to a boy was associated with high ACES (appraisals of cognition, emotion and situation) scores, and reliably predicted a tendency to value thoughts negatively – a behavioural trait more strongly associated with postpartum depression than the actual experiencing of a negative thought (Hall & Papageorgiou, 2005).

As for the socio-demographic risk factors, low maternal age, unemployment, being a single parent, pre-term delivery and no or little social support systems, contribute to low parenting competence, which has been shown to be an important mediating factor in the management of postpartum depression (Jones *et al.*, 2019; Martinez-Torteya *et al.*, 2018).

Finally, a history of depression before or during pregnancy significantly increases the risk for postpartum depression. One of the most influential studies supporting this association in postpartum depression is that of Robertson and colleagues (2004), who found that depression during pregnancy and a history of depression prior to pregnancy were two of the strongest predictors of postpartum depression in more than 20 000 postpartum women. In fact, a woman who has experienced a postpartum depressive episode, has between a 30 and 50 % risk of recurrence with subsequent pregnancies (American Psychiatric Association, 2013a). These findings are emphasised by Monroe and colleagues (2014) who reported the significant influence of a positive family history of depression with and without a traumatic life event. According to the American Psychiatric Association (2013b), heritability accounts for 40 % of depressive symptoms, and interestingly, the neuroticism personality trait contributes to a substantial portion of this genetic liability (Puyané *et al.*, 2022). Finally, and according to a recent meta-analysis of genome-wide association studies, although no single nucleotide polymorphism was associated with postpartum depression, significant correlations between postpartum depression, major depressive disorder, bipolar disorder, anxiety disorders, and polycystic ovary syndrome were identified (Guintivano *et al.*, 2023).

2.2 Aetiology of postpartum depression

Although the aetiology of postpartum depression is relevant to the understanding of the approved treatment options and the potential neurochemical effects on the developing brain (discussed later), the details thereof fall beyond the scope of the research question. Therefore, the aetiology of postpartum depression will be briefly discussed here, highlighting the most notable and relevant mechanisms. For a complete discussion, the reader is referred to detailed reviews (Maguire et al., 2020; Ming & Shinn-Yi, 2016; Payne & Maguire, 2019; Zonana & Gorman, 2005) on this topic.

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The exact mechanism of postpartum depression remains unknown but is thought to be due to the abrupt hormone withdrawal during the immediate postpartum period (Zonana & Gorman, 2005). Briefly, oestradiol and progesterone levels respectively rise to fifty and ten times the highest menstrual cycle levels during the third trimester of pregnancy, followed by a sudden drop shortly after parturition, and a return to normal levels within the first postpartum week (Bloch *et al.*, 2003). Such significant concentration changes, specifically that of oestrogen, can alter HPA function (Walf & Frye, 2006), which as alluded to earlier, is implicated in the pathophysiology of postpartum depression. To this end, HPA hyperactivity is also observed during pregnancy (Bloch *et al.*, 2003), and although its exact role remains unknown and debated (Meltzer-Brody *et al.*, 2011), elevated corticotropin-releasing hormone (CRH) has been proposed a diagnostic biomarker for postpartum depression (Yim *et al.*, 2009).

Traumatic life events or early-life adversity is known to influence HPA axis function, and to be a sufficient stressor to induce impaired maternal behaviours (Brummelte & Galea, 2010) and depressive-like behaviour in postpartum rodents (Roets *et al.*, 2023; Whitney *et al.*, 2023b). Moreover, antalarmin administration decreases depressive-like behaviour, via its CRH receptor antagonistic properties (Ducottet *et al.*, 2003). This is noteworthy, as the administration of oestradiol has been shown to induce antidepressant-like effects in ovariectomized rats (Bekku & Yoshimura, 2005; Galea *et al.*, 2001), while finasteride, a 5α-reductase inhibitor, induced depressive-like behaviour in female DBA/2J mice (Beckley & Finn, 2007) by preventing progesterone metabolism, and the consequent synthesis of the neurosteroid, allopregnanolone.

Allopregnanolone is of particular importance in postpartum depression, as it is the target of the only approved option for the treatment of postpartum depression (*see Section 2.4.1.1*). It is thought to induce its antidepressant effects by potentiating the gamma aminobutyric acid subtype A ($GABA_A$) receptor (Payne, 2021). The significant involvement of allopregnanolone in the pathophysiology of postpartum depression is supported by reports of decreased levels that correlate with depressive symptoms during the later stages of pregnancy (Hellgren *et al.*, 2014), which are reversed by the administration of the $GABA_A$ modulator, allopregnanolone (Zheng *et al.*, 2019). Moreover, a recent meta-analysis highlighted the significant role of dysfunctional GABAergic neurotransmission in postpartum depression (Guintivano *et al.*, 2023), re-affirming the role of allopregnanolone as a treatment option for postpartum depression.

Finally, and relevant to the current research question, is the role of neurotransmitters in the aetiology of postpartum depression. Although altered GABA, glutamate and dopamine concentrations are also implicated (Payne & Maguire, 2019), the role of serotonin is particularly relevant here. Hyposerotonergia is generally associated with depressive symptoms – an association supported by the therapeutic value of serotonergic-enhancing antidepressants in the treatment of postpartum (Appleby *et al.*, 1997; De Crescenzo *et al.*, 2014; Susser *et al.*, 2016) and non-postpartum (non-puerperal) depression (Hieronymus *et al.*, 2018; Locher *et al.*, 2017). Pre-clinically, decreased serotonergic neurotransmission has also been observed in models of postpartum depression (Qiu *et al.*, 2020; Roets *et al.*, 2023), and confirmed by rodent models of decreased serotonergic receptor expression (Lerch-Haner *et al.*, 2008) and related metabolic pathways (Anderson & Maes, 2013).

Taken together, the aetiology of postpartum depression, as highlighted here, is multifactorial with various neuro-biological pathways and processes (together with their downstream effects) implicated. Still, the treatment options used clinically, primarily target either GABAergic (brexanolone) or serotonergic neurotransmission (fluoxetine).

2.3 Signs and symptoms and the impact on mother-child relationships

The clinical signs and symptoms of postpartum depression are best summarized by the diagnostic criteria of major depressive disorder (non-puerperal depression), as set out in Table 2-1, below.

Table 2-1: Diagnostic criteria of major depressive disorder, according to the Diagnostic and statistical manual of mental disorders, 5th edition (DSM-V) (adapted from American Psychiatric Association, 2013b)

***five, or more, present most of the day, nearly every day during the same two-week period, and indicated by either the patient or observed by others*)**
Depressed mood.
Markedly diminished interest or pleasure in all, or almost all, activities.
At least one of the two mentioned symptoms above, must be present, together with any of the following:
Significant weight loss when not dieting or weight gain (more than 5 % of body weight in a month) or decrease/increase in appetite nearly every day.
Insomnia or hypersomnia.
Psychomotor agitation or retardation.
Fatigue or loss of energy.
Feelings of worthlessness or excessive inappropriate guilt.
Diminished ability to think or concentrate, or indecisiveness.
Recurrent thoughts of death (not just the fear of dying), recurrent suicidal ideation.
The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
The episode is not attributable to the physiological effects of a substance or to another medical condition.
The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.
There has never been a manic episode or a hypomanic episode.

The positive signs and symptoms, listed in Table 2-1 are then used to score the severity of the condition via different approved and validated questionnaires, such as the Hamilton Rating Scale for Depression, the Montgomery-Åsberg Depression Rating Scale, the Patient Health Questionnaire, or the Edinburgh Postnatal Depression Scale (Mughal *et al.*, 2022; Qaseem *et al.*, 2023), of which the latter is the most popular and also effective in diagnosing postpartum depression (Levis *et al.*, 2020; Park & Kim, 2023). Other postpartum-specific questionnaires include the Self-Reporting Questionnaire, the Zung Self-Rating Depression Scale and the Postpartum Depression Screening Scale (Mokwena, 2021). Of note, although not separately classified in the DSM-V or by the International Classification of Diseases-10 (ICD-10), the diagnostic criteria for postpartum depression is similar to that described above, with the only exception being that symptom onset (described in Table 2-1) is experienced or observed during the first four (American Psychiatric Association, 2013b) to six (World Health Organization, 2019) weeks after delivery. Importantly, symptom onset can also be during pregnancy (U.S. Food & Drug Administration, 2019). Screening for postpartum depression must therefore be considered and performed up to six months, postpartum (Mughal *et al.*, 2022).

As for the impact of these signs and symptoms (or positive diagnosis) on the mother-infant relationship, it is concerning that the decreased maternal interactions and bonding that is associated with postpartum depression can have serious and fatal consequences. To this end, it is unfortunately worth highlighting the devastating recent murder trial of Lauren Dickason, where these heart breaking consequences were broadcasted to the world (Davis, 2023). Unfortunately, neonaticide and infanticide is often associated with postpartum depression, yet is one of the worst documented causes of death, resulting in systematic data on this topic to be scarce (Spinelli, 2004). Early records do however suggest that an infant under the age of one, is killed daily in the United States, with this number considered to be a significant underestimation of the current global incidence (Overpeck *et al.*, 1998).

Other consequences of postpartum depression, as reviewed and extensively discussed by Mokwena (2021), include difficulty breastfeeding, which can augment the depressive symptoms by fuelling feelings (or perceptions) of being a “failed mother” (Beinschroth, 2020). Increased risk for child malnutrition, which can in turn adversely influence general health and childhood development is also related to postpartum depression, as are compromised emotional attachment to the child, and disrupted social and academic development of the child that increases the risk for the development of mental health conditions (juvenile depression) and impaired cognitive development (Netsi *et al.*, 2018). Alarmingly, these depressive symptoms (and their consequences) can last up to eleven years post-pregnancy, and negatively affect the immediate and broader family (Netsi *et al.*, 2018).

Considered together, accurate screening tools and diagnosis, together with increased awareness into the consequences of postpartum depression cannot be stressed enough. Moreover, appropriate social support structures and effective education can reduce the risk of postpartum depression, and where needed pharmacological treatment strategies can be considered.

2.4 Treating postpartum depression

2.4.1 General approach to treatment during the postpartum period

According to the most recent treatment guidelines, the American Psychological Association (2019) and College of Physicians (Qaseem *et al.*, 2023) recommend that antidepressant treatment for the general adult population must be initiated on either a psychotherapy strategy (i.e., behavioural with or without cognitive therapy, psychodynamic therapy, and supportive therapy) or a second-generation pharmacological antidepressant (i.e., selective serotonin re-uptake inhibitors; SSRIs). Interestingly, these panels of experts do not appear to prefer a specific class of antidepressants over another and recommend that prescribers must base their decision on the patient-specific nuances and risks for side effects. This approach is mainly due to larger comparative trials (Cipriani *et al.*, 2018), confirming the superiority of all classes of antidepressants over placebo treatment, and highlighting comparable between-class efficacy (Buelt & McQuaid, 2023). As to combining therapeutic interventions, the combination of cognitive behavioural therapy with a second-generation antidepressant (i.e., SSRI) is recommended only in patients with severe, persistent or recurrent symptoms (Buelt & McQuaid, 2023).

Importantly, despite these guidelines being intended for patients diagnosed with major depressive disorder (i.e., non-puerperal depression), the treatment options for women with postpartum depression are similar. Although no official guidelines, such as those mentioned above, exist for postpartum depression, SSRI drugs are generally recommended by clinicians (Dennis & Stewart, 2004). According to meta-analyses, SSRI drugs are superior to placebo controls for the treatment of postpartum depression and are also well tolerated (Brown *et al.*, 2021; Molyneaux *et al.*, 2015). However, the general consensus is that these studies, are generally small and heterogeneous, leading to low certainty of evidence (Brown *et al.*, 2021; Molyneaux *et al.*, 2015).

Because postpartum depressive symptoms can originate during pregnancy, pharmacological treatment can also be initiated during the antenatal period and continued into the postpartum phase. Therefore, studies evaluating the safety and efficacy of these drugs, exclusively in breastfeeding mothers, are scarce. Still, SSRI drugs are recommended while breastfeeding, with sertraline and paroxetine (and not fluoxetine) being the preferred options (Sie *et al.*, 2012). In this regard, perinatal fluoxetine treatment produced the highest proportion of infant plasma levels (22 %) of the antidepressants investigated (Weissman *et al.*, 2004). Intriguingly, although statistically comparable, infants only exposed to postnatal fluoxetine (i.e., via breast milk), had lower plasma levels than those exposed to the drug prenatally (i.e., *in utero*) (2.8 ng/ml *vs.* 7.9 ng/ml) (Weissman *et al.*, 2004). Still, uncertainty regarding the effects of these pharmacological drugs on the developing new-born, remains unknown and could discourage postpartum women from starting or completing pharmacological treatment regimens (Sachs *et al.*, 2013). Still, the risks of withholding antidepressant treatment during the peripartum period alluded to earlier, remains significant (van den Berg, 2020).

A great deal of uncertainty regarding appropriate and safe treatment strategies for postpartum depression exist. What is however important, is that pharmacological treatment options are not absolutely contraindicated during breastfeeding and can therefore be considered as viable options, as long as the individual drug nuances and risk profile of the patient are considered. Examples of pharmacological considerations, include the elimination half-life of the drug (and its metabolites), lipid solubility, and the bioavailability of the administered ingredient. Chemicals with prolonged half-lives, are more likely to accumulate in breast milk (Kelsey & Ward, 2020; Sachs *et al.*, 2013). High lipid-solubility and bioavailability properties allow the drug to readily cross the placenta and be expressed in breast milk, as well as increases the chances of the drug being absorbed by the infant (Heikkinen *et al.*, 2003; Kelsey & Ward, 2020; Sachs *et al.*, 2013). Other pharmacological characteristics, such as small molecular weight, and low volume of distribution and serum protein binding potential, all facilitate breast milk expression (Kelsey & Ward, 2020; Sachs *et al.*, 2013). Next, significant side effects, such as cardiac arrhythmias, would generally disqualify a treatment option in a breastfeeding mother. Conversely, the discontinuation of breastfeeding is also not advisable (although often considered), as early cessation is positively linked with postpartum depression, whereas exclusive breastfeeding may reduce depressive symptoms (Dias & Figueiredo, 2015; Figueiredo *et al.*, 2014; Watkins *et al.*, 2011). Finally, although the SSRI drugs are recommended by most experts, psychotherapy strategies must be considered as first-line options, where necessary and indicated (i.e., moderate depressive symptoms) (Dominiak *et al.*, 2021).

2.4.1.1 Brexanolone

Brexanolone is the only pharmacological treatment option, indicated for mild to severe postpartum depression, with Sage Therapeutics, Inc. receiving FDA[1]-approval in March 2019 (U.S. Food & Drug Administration, 2019). Currently, it is only available as an intravenous infusion, to be delivered over a total of 60 h, during which close monitoring of the patient required, because of excessive sedation and sudden loss of consciousness, associated with this drug. In fact, this recommendation is included in a Boxed Warning in the prescribing information leaflet (U.S. Food & Drug Administration, 2019).

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[1] *United States Food and Drug Administration*

Brexanolone acts as a positive allosteric modulator of the gamma aminobutyric acid subtype A ($GABA_A$) receptor, thereby mimicking the pharmacodynamic effects of allopregnanolone (Walton & Maguire, 2019). As mentioned previously, allopregnanolone levels of women either at risk of, or diagnosed with postpartum depression, have been reported to be decreased, relative to healthy controls (Hellgren *et al.*, 2014). That these levels were also inversely associated with symptom severity (Deligiannidis *et al.*, 2016), support the GABAergic deficit hypothesis of postpartum depression. Furthermore, the HPA axis is also regulated by GABAergic inhibition (Herman *et al.*, 2004), explaining why hypercortisolemia is implicated in the pathophysiology of postpartum depression (Melón *et al.*, 2018), and why brexanolone is effective in treating these neuro-biological shortfalls. Allopregnanolone (and by implication, brexanolone) also acts on the pregnane X receptors – a ligand-activated transcription factor of the nuclear receptors superfamily that regulate CYP3A gene expression, which plays a key role in the body's defence against foreign chemicals (or xenobiotics) (Kliewer *et al.*, 2002) and has been linked with anti-inflammatory properties (Li & Apte, 2015).

As to the efficacy of brexanolone in reversing postpartum depression symptoms, a recent meta-analysis by Zheng and colleagues (2019) found that not only were the antidepressant effects of a single brexanolone infusion, rapid, but these effects also lasted for a week. These effects also appear to be superior to both placebo treatment and SSRI-based regimens (Cooper *et al.*, 2019). The dosage form and administration route of brexanolone, practical and financial restrictions may become evident, and even prevent many women from receiving this treatment option. It is therefore worth highlighting that a cost-effectiveness model analysis, predicted that brexanolone treatment may actually be more affordable than an SSRI-regimen over an eleven year period (Eldar-Lissai *et al.*, 2020). Specifically, the total predicted medical costs for women treated with brexanolone was 7 745 USD (147 307 ZAR[1]) cheaper than an SSRI alternative regimen (65 908 *vs.* 73 653 USD) (Eldar-Lissai *et al.*, 2020).

Finally, brexanolone is expressed in breast milk, with peak levels (125 µg/l) observed between 24 and 48 h of infusion, at maximum dose. These levels, however, drop to below the limit of detection within three days after infusion, and the predicted median weight-adjusted percent of the maternal brexanolone dose, calculated as 0.69 % (Wald *et al.*, 2022). Considered together with the low oral bio-availability of brexanolone, the risk for associated side effects in the infant is reduced, allowing the mother to continue breastfeeding whilst receiving brexanolone treatment (LactMed®, 2023b).

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[1] *Calculated on the exchange rate of 18 September 2023 (1 USD = 19.02 ZAR).*

2.4.1.2 Fluoxetine

Fluoxetine is one of the most researched and well-known SSRI drugs available. It prevents the reuptake of serotonin into the neuron, by inhibiting the serotonin transporter, resulting in increased synaptic serotonin levels (DeBattista, 2021). Fluoxetine ($t_{½}$ = 53 h) is highly protein bound (94 %) and hepatically metabolized by *N*-demethylation to its active metabolite, norfluoxetine ($t_{½}$ = 240 h) (Heikkinen *et al.*, 2003; O'Donnell *et al.*, 2018; van den Berg, 2020) which is just as potent as fluoxetine (Heikkinen *et al.*, 2003) and results in prolonged pharmacodynamic effects, even after cessation of treatment (DeBattista, 2021). Because of its moderately potent CYP2D6 inhibiting properties, fluoxetine can decrease the metabolism of other co-administered drugs (i.e., carbamazepine, haloperidol, metoprolol, tamoxifen etc.) that are substrates of this specific pathway (van den Berg, 2020). From a safety perspective, fluoxetine is associated with moderate gastrointestinal (nausea, vomiting and/or diarrhoea) and sexual (inhibited arousal, libido and/or orgasm) side effects, and to a lesser extent agitation, sedation, seizures, weight gain and cardiac-related effects (O'Donnell *et al.*, 2018). However, when compared to other antidepressants, fluoxetine (together with agomelatine, citalopram, escitalopram, sertraline, and vortioxetine) appears to be best tolerated (Cipriani *et al.*, 2018).

Although not FDA[1]-approved, fluoxetine remains one of the most popular drugs to treat postpartum depression (Cipriani *et al.*, 2018). As to infant exposure via passive administration (i.e., via breast milk), data suggest that fluoxetine and norfluoxetine breast milk concentrations peak around 8 to 9 hours after maternal dosing (Hendrick *et al.*, 2001), and are relatively low (yet higher than other SSRIs), thereby reducing the overall risk for drug-induced effects (Gao *et al.*, 2018; Heikkinen *et al.*, 2003). Various studies (Berle *et al.*, 2004; Heikkinen *et al.*, 2003; Kim *et al.*, 2006; Oberlander *et al.*, 2005; Taddio *et al.*, 1996; Weissman *et al.*, 2004) have quantified these concentrations, with the average maternal weight-adjusted dose to which the infant was exposed, across these studies, calculated as 3.96 ± 2.25[2] % [2.07; 5.84[3] %]. To this point, although infant plasma levels of fluoxetine and norfluoxetine have been measured (Epperson *et al.*, 2003; Heikkinen *et al.*, 2003), the exact concentration that reaches the developing brain, remains unknown. Infant adverse effects that are associated with passively administered fluoxetine, include irritability, poor feeding, colic, fussiness, drowsiness and reduced growth (Heikkinen *et al.*, 2003; LactMed®, 2023a). It is however worth noting that no impaired developmental effects were found in these infants, after a one-year follow-up study, supporting

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[1] *United States Food and Drug Administration*

[2] *Mean ± standard deviation.*

[3] *Upper and lower limit of the 95 % confidence interval of the mean.*

the continuation of fluoxetine treatment while breastfeeding (LactMed®, 2023a). If, however, the patient or prescriber is concerned, other SSRI drugs (i.e., sertraline) that are less expressed in the breast milk could be considered (Weissman *et al.*, 2004).

2.5 Pharmacological treatment options and the impact on the developing infant brain

2.5.1 Juvenile brain development

What we know about juvenile brain development, is largely based on pre-clinical (i.e., animal) studies. It is therefore important to keep in mind that the developmental process, although comparable between species, contains species-specific nuances (Dobbing & Sands, 1979; Murrin *et al.*, 2007), such as brain weights of rats at birth being comparable to that of humans at the end of the second trimester (Murrin *et al.*, 2007). To place the developmental process of the rat brain into context with that of its human counterpart, it should be noted that prenatal rodent development occurs over a 21-day gestational period, whereas human development occurs over a period of forty weeks. As previously described by Steyn (2018) and summarized in Figure 2-1, the prenatal developmental period of the rat resembles the first two trimesters of the human process, with the end of the third trimester, being comparable to the development during the first three weeks of a rat pup's life (Kepser & Homberg, 2015). The onset of puberty and start of adolescence in humans is generally accepted to be between twelve and eighteen years of age (Spear, 2007), however, genetics, environment and nutrition may influence the onset thereof. This developmental phase is similar to that of the 35-day old rat (Drzewiecki *et al.*, 2020; Murrin *et al.*, 2007), with adulthood accepted to be from PND60, onwards (Malkesman & Weller, 2009).

In rats, serotonergic neurons are detected first (in the 8 mm embryo), with noradrenergic neurons only detected later (11 mm embryo) (Golden, 1982). By gestational day 10, serotonin transporter mRNA is already detectable (Hansson *et al.*, 1998), with the noradrenergic neurons only starting to differentiate (Lauder & Bloom, 1974; Thomas *et al.*, 1995). Even before this (i.e., gestation day 10), peak levels of tryptophan hydroxylase (the enzyme responsible for serotonin synthesis) is detected (Rho & Storey, 2001), whilst tyrosine hydroxylase, the rate-limiting enzyme responsible for noradrenaline synthesis, is only detectable at gestational day 15 (Chugani *et al.*, 1999; Kato *et al.*, 1982; Murrin *et al.*, 2007). Between gestational days 14 and 17, the majority of brain neuro- and synaptogenesis in most brain regions, take place (Green *et al.*, 1999). As differentiation of the neurons are now already underway, projections of the serotonergic and noradrenergic neurons, reach the cortex by gestational days 17 and 18 (Lauder & Bloom, 1974; Markus & Petit, 1987; Wallace & Lauder, 1983) with the serotonergic neurons already representing adult distributions by gestational day 19 (Aitken & Törk, 1988; Wallace & Lauder, 1983).

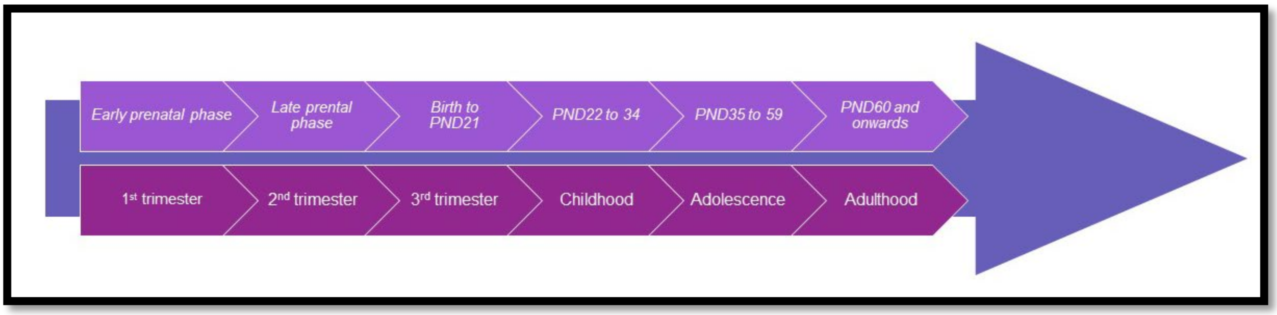

Figure 2-1: Summarized comparison of rodent and human developmental phases
A comparison of the different developmental phases of humans and rodents, giving insight into the comparability between species, and aiding the interpretation and translation of pre-clinical (animal) to clinical (human) data (adapted from Kepser & Homberg, 2015; Malkesman & Weller, 2009), and reproduced with the permission of Steyn (2018).
PND: *Postnatal day.*

At the time of birth, serotonergic receptor density in the rat brain is at its highest (Daval *et al.*, 1987; Murrin *et al.*, 2007), and includes a functional serotonergic reuptake system (Enjalbert *et al.*, 1978; Nelson *et al.*, 1980; Tissari, 1975). In contrast, noradrenergic receptor expression is only observed from PND01, onwards (Winzer-Serhan & Leslie, 1997; Winzer-Serhan *et al.*, 1996b), with α_{2A} mRNA and low levels of α_1 adrenergic receptors being detected around the time of birth (Morris *et al.*, 1980; Winzer-Serhan *et al.*, 1996a).

By PND21, the serotonergic reuptake system is already functioning, whereas detectable levels of noradrenergic reuptake transporters, emerge on PND05, peaking by PND20 (Murrin *et al.*, 2007) when α_1 receptor expression mirrors that of adults (Morris *et al.*, 1980). The growth of serotonergic dendrites on PND07 (Lidov & Molliver, 1982; Loizou & Salt, 1970) is so significant, that administration of 8-hydroxy-2-(di-n-propylamino)-tetralin (a 5-HT_{1A} agonist), induces serotonin syndrome-associated behaviour in Sprague-Dawley pups (Darmani & Ahmad, 1999). Two weeks after birth, 5-HT_2 receptor densities exceed that of the adult rat, followed by a gradual decline (Roth *et al.*, 1991). That a 5-$HT_{2A/C}$ agonist already induces “wet dog shakes” in pre-pubertal rats (Darmani & Ahmad, 1999), confirms the functionality of these receptors. Also on PND14, a significant increase in striatal tyrosine hydroxylase concentrations is observed, indicating further development of the noradrenergic nervous system (Murrin *et al.*, 2007), and is supported by an increase in noradrenergic neuron firing rates (Nakamura *et al.*, 1987). Noradrenergic receptor densities significantly increase and reach adult levels by PND15 (Happe *et al.*, 2004; Harden *et al.*, 1977; Morris *et al.*, 1980; Pittman *et al.*, 1980; Slotkin *et al.*, 1990) at which time the overall synaptogenesis of the noradrenergic and serotonergic systems are 55 % and 75 %, respectively, completed (Lauder & Bloom, 1975). Interestingly, by PND18 the noradrenergic system in the cerebellum and brainstem reaches maturity (Konkol *et al.*, 1978), however, this is an exception to the general rule that the noradrenergic system matures after the serotonergic one. At three weeks of age, the serotonergic dendrite network is already comparable to that of the adult rat brain (Dori *et al.*, 1996; Lidov & Molliver, 1982; Loizou & Salt, 1970).

Starting on PND21, serotonin concentrations start to slowly decrease towards adult concentrations (Loizou, 1972; Whitaker-Azmitia, 2005), suggesting maturity. At the same time, a significant increase in tyrosine hydroxylase concentrations in the cortex is observed, emphasising noradrenergic maturation (Murrin *et al.*, 2007). In support, noradrenergic α_2 receptor stimulation is at its highest during the fourth postnatal week (Happe *et al.*, 1999). By PND35 the noradrenergic system has reached maturity (Konkol *et al.*, 1978; Loizou & Salt, 1970; Morris *et al.*, 1980; Murrin *et al.*, 2007), although one report suggests noradrenergic synapses to only reach adulthood by PND60 (Markus & Petit, 1987). Regardless, the general consensus is that the serotonergic system matures before the onset of puberty, whilst the adult similarity of the noradrenergic system is only reached with or after pubertal onset (Murrin *et al.*, 2007). This would therefore sensitize the developing infant brain to the neurochemical influence of a serotonergic-enhancing drug, such as fluoxetine.

2.5.2 Bio-behavioural effects of early-life exposure to antidepressants

As mentioned previously, both brexanolone and fluoxetine are expressed in the breast milk. However, because of the low oral bioavailability of brexanolone, the risk for associated side effects in the infant appears to be low (LactMed®, 2023b). In contrast, and largely because of the prolonged elimination half-life of norfluoxetine, together with the significant concentrations expressed in the breast milk (Weissman *et al.*, 2004), infant irritability, poor feeding, colic, fussiness, drowsiness and reduced growth are associated with passively administered fluoxetine (Heikkinen *et al.*, 2003; LactMed®, 2023a).

Therefore, due of ethical and practical reasons, the long-term bio-behavioural effects, and mechanisms of neurodevelopmental fluoxetine exposure, remains unknown. Hence, a review of the available pre-clinical data in this regard is invaluable. Importantly, studies exclusively investigating the postnatal exposure to fluoxetine (via breast milk) in offspring are scarce, with the majority of studies generally investigating fluoxetine administration during the extended gestation (including the early postpartum) period.

Maternal (during gestation and lactation) fluoxetine (5 mg/kg/day) did not delay pubertal onset in Wistar rat offspring, as indicated by oestrogen and testosterone plasma concentrations. Moreover, plasma corticosterone levels of pups exposed to fluoxetine during gestation and lactation was also unaffected, as was body weight (Barbosa *et al.*, 2019). Maternal fluoxetine (5 mg/kg/day during gestation and lactation periods) also induced long-lasting neurochemical effects, with hippocampal methylation of Wistar offspring being altered on PND22 (Toffoli *et al.*, 2014), and lasting into adulthood (Silva *et al.*, 2018). Although anxiety-like behaviour was unaffected by maternal fluoxetine in both juvenile (Toffoli *et al.*, 2014) and adult rats (Houwing *et al.*, 2019; Silva *et al.*, 2018), social behaviour was decreased during adulthood (Silva *et al.*, 2018), while pain sensitivity increased during pre-pubertal development (Toffoli *et al.*, 2014). In terms of depressive-like behaviour and anhedonia, maternal fluoxetine (10 mg/kg/day during gestation and lactation) did not induce any robust alterations in these behavioural parameters of Wistar rats (Houwing *et al.*, 2019) but it must be noted that the differences that were observed by Houwing and colleagues (2019) were dependent on sex and serotonin transporter expression.

Studies that have however investigated the effects of fluoxetine exposure, exclusively during the lactation period, reported less significant findings. For instance, de Andrade Silva and colleagues (2023) observed that Wistar pups, exposed to passively administered fluoxetine (10 mg/kg/day administered to dams) from PND01 to PND21, gained less weight, yet presented with improved hippocampal mitochondrial function (NAD^+/NADH)[1] and decreased antioxidant defences (GSH/GSSG). Hippocampal gene expression related to serotonin, and a marker of neuroplasticity (brain-derived neurotrophic factor; BDNF), were however unaffected by passively administered fluoxetine (de Andrade Silva *et al.*, 2023), as was oestrous cyclicity (Ray *et al.*, 2014). Even at escalating doses (1, 10 and 20 mg/kg) of fluoxetine, Zaccarelli-Magalhães and colleagues (2020) observed no behavioural (locomotor and social interactions) effects in Wistar offspring exposed to passively administered fluoxetine, irrespective of dose. In contrast, studies where pups were exposed to acute doses of passively administered fluoxetine, reported increased anxiety-like behaviour later in life together with reduced social behaviour (Olivier *et al.*, 2011).

These effects are somewhat mirrored in clinical studies. For example, infants (younger than six months) that were exposed to fluoxetine via the breast milk (mothers receiving 20 to 40 mg oral fluoxetine, daily), did not show any changes in plasma serotonin levels (Epperson *et al.*, 2003), nor were there any neurological and growth deficits after a one year follow up study in infants exposed to passively administered fluoxetine in relation to controls (Heikkinen *et al.*, 2003). An interesting observation by Hendrick and colleagues (2001), noted that infant plasma fluoxetine and norfluoxetine concentrations were less likely to be detected at daily maternal doses lower than 20 mg. This is noteworthy, as fluoxetine is started at this dose during a period when glomerular filtration rate and metabolic activities are increased, that could result in reduced

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[1] *Nicotinamide adenine dinucleotide in its oxidized (NAD^+) and reduced (NADH) forms.*

plasma levels (Lee & Burgees, 2020), and explain the general side effects mentioned earlier, that have been reported with fluoxetine during the lactation period. In support of the indicated use of fluoxetine while breastfeeding, Gao and colleagues (2018) found that in a meta-analysis of more than nine million births, available evidence suggests that although a small risk of congenital malformation is associated with fluoxetine (and other SSRIs) during the peripartum period, no significant increase in risk was observed, when the results were restricted to women with psychiatric diagnosis.

2.6 The Flinders sensitive line rat as a suitable rodent model

As first described by Russel and Overstreet (1987), the Flinders sensitive line (FSL) was originally bred as a model to be resistant to the organophosphate, diisopropylfluorophosphonate. Instead, a more sensitive rat line was created, displaying several characteristics resembling a depressive-like phenotype. Importantly, the Flinders resistant line (FRL) rat is the appropriate control for the FSL rat and is only more resistant to the organophosphate effects, relative to its FSL counterpart (Russell & Overstreet, 1987). The behavioural deficits, alluded to, displayed by the FSL rat include increased behavioural despair (Abildgaard *et al.*, 2017; Oberholzer *et al.*, 2018; Roets *et al.*, 2023; Tillmann & Wegener, 2019; Uys *et al.*, 2017; Wei *et al.*, 2015), and impaired cognitive function (du Jardin *et al.*, 2016; Oberholzer *et al.*, 2018; Tillmann *et al.*, 2019; Uys *et al.*, 2017), relative to treatment naïve FRL controls. As for the construct validity of this model, the FSL rat presents with serotonergic (Hvilsom *et al.*, 2019; Roets *et al.*, 2023; Whitney *et al.*, 2023b) and noradrenergic (Roets *et al.*, 2023; Whitney *et al.*, 2023b) neurotransmission profiles, akin to the clinical picture of depression. In addition, markers of neuroplasticity are also decreased in this specific strain (Melas *et al.*, 2013; Stiernborg *et al.*, 2022; Wei *et al.*, 2015), whilst oxidative stress (Mohideen *et al.*, 2021; Whitney *et al.*, 2023b) and markers of inflammation (Abildgaard *et al.*, 2017; Wei *et al.*, 2016) are increased. Indications of mitochondrial dysfunction (a cause of increased oxidative stress damage) has also been inferred for this strain (Chen *et al.*, 2013; Whitney *et al.*, 2023a, 2023b) – thereby increasing the validity of it for neuropsychiatric research. Notably, these bio-behavioural deficits are also observed during the early postpartum period (Oosthuizen, 2022; Roets *et al.*, 2023)

In terms of the predictive validity of this strain, the mentioned bio-behaviour deficits are reversed by established pharmacological and non-pharmacological strategies (Kanemaru *et al.*, 2008; Mncube & Harvey, 2022; Overstreet *et al.*, 2004; Steyn *et al.*, 2018; Steyn *et al.*, 2020; Whitney *et al.*, 2023a). Taken together, these characteristics, confirm the FSL rat as an accepted rodent model of depression with strong face (increased behavioural despair behaviour), construct (altered monoaminergic and redox state) and predictive validity (reversal of bio-behavioural deficits with clinically approved antidepressants).

As to whether the FSL rat is a suitable model for juvenile depression, the FSL rat (irrespective of sex) weighs more than FRL controls, and presents with increased locomotor activity on postnatal day 21 (PND21), that could mimic psychomotor agitation (Whitney *et al.*, 2023b) – two diagnostic signs and symptoms of juvenile depression (American Psychiatric Association, 2013b). At pubertal onset (between PND35 and 43) (Drzewiecki *et al.*, 2020; Murrin *et al.*, 2007), Whitney and colleagues (2023b) reported that male and female FSL rats already displayed behaviour akin to behavioural despair (increased time spent immobile and decreased swimming and struggling). Such behaviour was accompanied by reduced whole brain weight, and increased hippocampal noradrenaline levels, serotonin turnover and oxidative stress – findings that are supported by others (Malkesman & Weller, 2009; Malkesman *et al.*, 2007; Malkesman *et al.*, 2006).

Considered together, the FSL rat is a suitable and appropriate model for the current study, as the face validity characteristics of this strain, can be translated to the human condition, where genetic susceptibility plays a significant role in the mental health of the offspring (2013b). Moreover, that these rats present with a depressive-like phenotype during the postpartum period (Roets *et al.*, 2023), makes it a translatable model to investigate the effects of passively administered fluoxetine in offspring. Finally, that prepubertal, male and female FSL rats also present with serotonergic and oxidative stress deficits, not only makes it a valuable model in terms of genetic predisposition, but also to identify any significant neurochemical alterations, caused by the passively administered fluoxetine.

CHAPTER 3: MATERIALS AND METHODS FOR NEUROCHEMICAL ANALYSES

This chapter gives a brief description of the analytical methodology used to measure the different neurochemical markers and drug concentrations, as well as data relevant to the validation and reproducibility of the method used. In addition, a brief description of where the samples analysed in this study was sampled from is also given.

3.1 Where tissue was sampled from

Animals were bred, supplied by and housed at the DSI/NWU Vivarium (SAVC reg: FR15/13458; SANAS GLP compliance: G0019; AAALAC accreditation file: #1717) of the Pre-Clinical Drug Development Platform of the North-West University. Adult female rats were individually housed with their litter until PPD21 (ethics approval number: NWU-00434-21-A5). No adverse events were reported during the initial study (Oosthuizen, 2022). The analysed tissue was divided into different experimental groups, with an even distribution of male and female samples. All bio-analyses were performed on the liquid chromatography/mass spectrometry (LC-MS) as described below. Importantly, the methods described below used in recent publications (Oosthuizen, 2022; Whitney *et al.*, 2023a, 2023b). To contribute to in-house transparency and validation, as well as interlaboratory reproducibility, a detailed description (including regression line details and specifications of altered analytical settings) of the method used, is also included.

3.2 Sample preparation

Whole brain samples were removed from the -80 °C fridge and cut into small pieces, whereafter it was weighed, and 0.5 ml of the Prep solution added to the sample. The Prep solution (0.1 % (*v/v*) formic acid) contained 200 ng/ml escitalopram as internal standard and 1:1 MeOH: Acetonitrile (ACN). This sample and 0.5 ml Prep solution was homogenized by sonication (twice for 12 s, at an amplitude of 14 µ; MSE ultrasonic disintegrator, Nuaillé, FRA), whereafter another 0.5 ml Prep solution was added. The mixture was left on ice for 20 min to complete protein precipitation and then centrifuged at 20 817 rcf for 25 min at 4 ºC. Next, 0.3 ml of the supernatant was transferred to an amber sample vial, that was used for LC-MS analysis. The Ultivo Triple Quadrupole LC-MS System, controlled by the MassHunter software from Agilent Technologies, Inc. (Santa Clara, USA) was used for analyses. Fluoxetine (FLX), norfluoxetine (nFLX), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and oxidized (GSSG) and reduced (GSH) glutathione concentrations were determined in the whole brain samples.

Results obtained were converted from ng/ml of the injected sample to ng/g of the wet brain tissue weight.

3.3 Method validation

3.3.1 Standard solutions

Approximately 1 mg of each analyte was dissolved separately in 10 ml of a 10 % MeOH solution in amber volumetric flasks. From the stock solutions of each analyte, a combined serial dilution series consisting of six to seven concentrations were prepared to construct a standard calibration curve and determine the linear range of each metabolite.

3.3.2 Internal standard solution

A stock solution of the internal standard, escitalopram, was prepared at a concentration of 200 ng/ml using a solvent mixture of 0.1 % (*v/v*) formic acid and 1:1 MeOH: Acetonitrile (ACN). This working solution was also used for the preparation of the different biological sample matrices and standards.

3.3.3 Mobile phase

A gradient mobile phase consisting of (A) 0.1 % formic acid (FA) and LC-MS grade water and (B) 0.1 % formic acid and ACN was prepared. Table 3-1 indicates how the gradient elution of the mobile phase was applied. The 3 min post-time was allowed to reset to the starting condition of mobile phase mixture between (A) and (B).

Table 3-1: Mobile phase gradient setup
The mobile phase was made up of HPLC water, 0.1 % (v/v) formic acid (FA) and 0.1 % acetonitrile (ACN), with a 3-minute post-time running period included.

Step	**Time (*min*)**	**A (%)** ***HPLC water / 0.1 % FA***	**B (%)** ***ACN / 0.1 % FA***
1	Start condition 0	95.0	5.0
2	3.0	95.0	5.0
3	4.3	0.0	100.0
4	12.0	0.0	100.0
5	14.0	95.0	5.0
6	15.0	95.0	5.0
7	Post-time 3	95.0	5.0

3.3.4 Multiple reaction monitoring optimisation

A 1 mg/ml solution of each analyte was used to optimise ionisation and to determine the optimum settings for the detection product ions. Table 3-2 represents the optimum MS settings for each metabolite precursor to qualify and quantify product ions.

Table 3-2: Optimum instrument settings for the identification and quantification of product ions
5-HIAA: *5-hydroxyindoleacetic acid.* ***5-HT:*** *5-hydroxytryptamine.* ***ESC:*** *Escitalopram (internal standard).* ***FLX:*** *Fluoxetine.* ***GSH:*** *Glutathione.* ***GSSG:*** *Glutathione disulphide.* ***MRM:*** *Multiple reaction monitoring.* ***nFLX:*** *Norfluoxetine.* ***RRT:*** *Relative retention time.*

LC instrument settings							
			Flow rate	0.3 ml/min			
			Injection volume	2 µl			
			Run time	18 min (15 min plus 3 min post-time)			
Mass spectrometer settings							
			Source parameter	Positive value			
			Gas temperature	350 °C			
			Gas flow	13 l/min			
			Nebulizer	60			
			Capillary voltage	4000 V			
Analyte setup							
Analyte	***Transition (m/z)***	***Dwell (ms)***	***Fragmentor (V)***	***Collision energy (V)***	***Polarity***	***Scan***	***RRT (min)***
5-HIAA	192.1 ≥ 145.9	100	61	17	Positive	MRM	± 8.446
5-HT	177.1 ≥ 160.1		56	13			± 2.196
FLX	310.1 ≥ 148.1		51	5			± 9.201
GSH	308.1 ≥ 179.0		76	9			± 1.702
GSSG	613.2 ≥ 231.0		121	37			± 1.692
nFLX	296.1 ≥ 74.0		51	169			± 9.144
I.Std (ESC)	325.2 ≥ 108.9		76	53			± 8.963

3.3.5 Linearity and calibration curve

Linearity was achieved by preparing six to seven standard concentrations of each analyte measured (Table 3-3). Three replicates of each concentration of the standards range were injected onto the LC-MS system to establish linear regression. According to Shabir (2005), the linear regression value (R^2) of the calibration curves must not be less than 0.95.

3.3.6 Quantification and detection limits

The limits of quantification (LOQ) and detection (LOD) were defined as the minimum concentration where the signal-to-noise ratio was at least 10:1 (LOQ) and 3:1 (LOD) greater than the average background noise of a non-spiked blank sample (a sample only containing the internal standard) at the retention time of each analyte, respectively (Desimoni & Brunetti, 2015; Shrivastava & Gupta, 2011).

3.3.7 Precision and accuracy

Four concentrations of each analyte, ranging from low to high, were chosen and three determinations for each concentration were performed to establish precision and accuracy. Precision results were expressed in percentage relative standard deviation (% RSD) from the mean and the acceptability criterion for each concentration level was not to exceed 15 % (Shabir, 2005; U.S. Food & Drug Administration, 2018). The accuracy results for each concentration were determined by comparing the closeness of the mean test concentration result to that of the true concentration value. The accuracy results were expressed as percentage recovery. The acceptability criterion for accuracy was to fall between 90 % to 110 % for each concentration (Shabir, 2005; U.S. Food & Drug Administration, 2018; van de Merbel, 2008).

3.3.8 Stability

Stability was determined by injecting the six standard solutions and prepared samples twice onto the LC-MS system over 24 h. The initial injection set was assayed immediately and served as the respective reference values at time zero with a 100 % stability. Samples were left at room temperature in the autosampler tray and reinjected after 24 h. The stability of each analyte was expressed as the mean percentage stability ± standard deviation, at 24 h.

3.4 Method validation results

The calibration curves constructed were evaluated by means of interpreting respective linear regression values. Linearity was excellent over the respective calibration ranges (Table 3-3), with the corresponding coefficient of determination (R^2) values consistently greater than 0.9999. Moreover, the coefficient of variation for the internal standard was also less than 10 %.

Table 3-3: Linear regression line equation and coefficient of determination (R^2)
All calibration curves and linearity were calculated in GraphPad Prism (version 10). ***% CV:*** *Coefficient of variation.* ***5-HIAA:*** *5-hydroxyindoleacetic acid.* ***5-HT:*** *5-hydroxytryptamine.* ***ESC:*** *Escitalopram (internal standard).* ***FLX:*** *Fluoxetine.* ***GSH:*** *Glutathione.* ***GSSG:*** *Glutathione disulphide.* ***LOD:*** *Limit of detection.* ***LOQ:*** *Limit of quantification.* ***nFLX:*** *Norfluoxetine.*

Analyte	Concentration range	Linear regression equation	R^2	Is slope significantly non-zero?
5-HIAA	7.8125; 15.625 31.25; 62.5 125; 250 500 ng/ml	$y = 8.01x - 170$	0.9999	Yes $p \leq 0.0005$
5-HT		$y = 131x - 2334$		
FLX		$y = 7.50x - 119$		
nFLX		$y = 1.49x - 32.1$		
GSH	1.25; 2.5; 5.0; 10.0; 20.0; 30.0 µg/ml	$y = 4446x - 1498$		
GSSG		$y = 95.1x - 167$		
I.Std (ESC)	200 ng/ml	***Mean response = 18 402 ± 1 818; % CV = 9.88 %***		

3.4.1 Quantification and detection limits

The limits of quantification (LOQ) and detection (LOD) were determined by means of mathematical formula, the signal to noise approach, and also on-column with a 2 µl injection volume for all the analytes (Table 3-4).

Table 3-4: Limits of quantification and detection results
5-HIAA: *5-hydroxyindoleacetic acid.* ***5-HT:*** *5-hydroxytryptamine.* ***FLX:*** *Fluoxetine.* ***GSH:*** *Glutathione.* ***GSSG:*** *Glutathione disulphide.* ***LOD:*** *Limit of detection.* ***LOQ:*** *Limit of quantification.* ***nFLX:*** *Norfluoxetine.*

Analyte	Calculation approach		Signal to noise approach	
	LOQ	***LOD***	***LOQ***	***LOD***
5-HIAA (*ng/ml*)	115.03	37.96	7.8125	3.90625
5-HT (*ng/ml*)	93.65	30.90	7.8125	3.90625
FLX (*ng/ml*)	100.49	33.16	15.625	7.8125
nFLX (*ng/ml*)	99.75	32.92	15.625	7.8125
GSH (*µg/ml*)	1.51	0.49	0.3125	0.3125
GSSG (*µg/ml*)	3.43	1.13	0.625	0.625

3.4.2 Precision and accuracy

The precision and accuracy results of the four tested concentrations of each analyte are provided in Table 3-5. Both precision and accuracy results were within the acceptable criteria ranges set by the method validation parameters. For all four concentrations of the respective analytes, the percentage RSD (relative standard deviation) for both intra- and inter-sample precision was below the required 15 % (*Intra* = 5.21 ± 3.51 % [3.7; 6.7 %]; *Inter* = 4.96 ± 4.32[1] % [3.1; 6.8[2] %]). Inter-sample values were unfortunately not calculated and must be determined in prospective studies using this method. The accuracy of all concentration levels for all the analytes tested was between 84 % and 109 % (mean value = 94.4 ± 5.69[3] % [94.0; 98.8[4] %]).

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[1] *Mean ± standard deviation.*

[2] *Upper and lower values of the 95 % confidence interval of the mean.*

[3] *Mean ± standard deviation.*

[4] *Upper and lower values of the 95 % confidence interval of the mean.*

Table 3-5: Accuracy and precision results
5-HIAA: *5-hydroxyindoleacetic acid.* ***5-HIAA:*** *5-hydroxyindoleacetic acid.* ***5-HT:*** *5-hydroxytryptamine.* ***FLX:*** *Fluoxetine.* ***GSH:*** *Glutathione.* ***GSSG:*** *Glutathione disulphide.* ***nFLX:*** *Norfluoxetine.* ***RSD:*** *Relative standard deviation.*

[Analyte]	**Intra-sample (*n* = 3)**			**[Analyte]**	**Inter-sample (*n* = 3)**		
	Measured c	***Precision (% RSD)***	***Accuracy (%)***		***Measured c***	***Precision (% RSD)***	***Accuracy (%)***
5-HIAA (*ng/ml*)							
7.8125	*6.59*	*11.78*	84.32	***7.8125***	*6.19*	*14.35*	79.35
15.625	*15.13*	*2.56*	96.84	***15.625***	*14.67*	*0.76*	93.94
31.25	*30.40*	*9.30*	97.29	***31.25***	*29.39*	*6.49*	90.87
62.5	*63.93*	*8.07*	102.28	***62.5***	*65.03*	*9.14*	104.05
5-HT (*ng/ml*)							
7.8125	*6.68*	*2.40*	85.62	***7.8125***	*6.37*	*2.62*	81.65
15.625	*15.16*	*0.86*	97.03	***15.625***	*14.91*	*1.34*	95.46
31.25	*32.73*	*2.85*	104.74	***31.25***	*33.10*	*3.56*	105.94
62.5	*67.95*	*1.09*	108.72	***62.5***	*70.38*	*2.27*	112.62
FLX (*ng/ml*)							
7.8125	*7.32*	*6.92*	93.78	***7.8125***	*6.93*	*4.87*	88.78
15.625	*15.43*	*5.22*	98.75	***15.625***	*14.99*	*5.01*	95.94
31.25	*29.83*	*4.42*	95.45	***31.25***	*26.99*	*2.73*	86.39
62.5	*60.09*	*1.34*	96.15	***62.5***	*56.58*	*2.61*	90.52
nFLX (*ng/ml*)							
7.8125	*7.31*	*9.58*	93.57	***7.8125***	*7.71*	*15.73*	98.75
15.625	*15.80*	*4.43*	101.18	***15.625***	*14.59*	*4.80*	93.41
31.25	*30.78*	*7.30*	98.50	***31.25***	*28.35*	*14.39*	90.73
62.5	*61.54*	*4.55*	98.46	***62.5***	*58.30*	*4.33*	93.28
GSH (*µg/ml*)							
1.25	*1.22*	*1.04*	98.04	***1.25***	*1.20*	*2.98*	96.51
2.5	*2.41*	*6.44*	96.41	***2.5***	*2.45*	*2.67*	96.85
5.0	*4.96*	*4.42*	99.33	***5.0***	*4.90*	*1.47*	98.02
10.0	*9.64*	*4.35*	96.42	***10.0***	*9.44*	*1.27*	94.43
GSSG (*µg/ml*)							
1.25	*1.19*	*2.00*	95.82	***1.25***	*1.12*	*2.57*	89.55
2.5	*2.10*	*2.74*	84.06	***2.5***	*2.09*	*2.32*	83.70
5.0	*4.90*	*13.81*	98.01	***5.0***	*4.59*	*7.74*	91.84
10.0	*9.38*	*7.52*	93.83	***10.0***	*8.87*	*2.89*	88.76

3.4.3 Percentage recovery and stability

The mean absolute recovery for each analyte measured in triplicate for all four tested concentrations was constantly above 90 % with the mean recovery indicated in Table 3-6. All the analytes were found to be stable within a 48-h period in the autosampler at room temperature.

Table 3-6: Percentage recovery results

Where applicable data are presented as mean ± standard deviation and range of results. ***5-HIAA:*** *5-hydroxyindoleacetic acid.* ***5-HT:*** *5-hydroxytryptamine.* ***FLX:*** *Fluoxetine.* ***GSH:*** *Glutathione.* ***GSSG:*** *Glutathione disulphide.* ***nFLX:*** *Norfluoxetine.*

Analyte	Concentration	Recovery (%) (n = 3)	
		mean ± SD	Range
5-HIAA (*ng/ml*)	7.8125	90.00 ± 13.33	76.7 – 103.3
	31.25	89.09 ± 8.67	82.1 – 98.8
	125	98.89 ± 3.14	95.3 – 101.2
5-HT (*ng/ml*)	7.8125	100.58 ± 2.51	98.3 – 103.3
	31.25	94.15 ± 2.71	91.3 – 96.7
	125	98.05 ± 1.45	96.4 – 98.9
FLX (*ng/ml*)	7.8125	103.45 ± 11.95	89.7 – 110.3
	31.25	101.47 ± 4.98	96.7 – 106.6
	125	96.71 ± 2.91	93.4 – 98.7
nFLX (*ng/ml*)	7.8125	83.33 ± 7.22	75.0 – 87.5
	31.25	89.66 ± 7.65	86.2 – 96.6
	125	99.69 ± 6.57	95.3 – 103.8
GSH (*µg/ml*)	1.25	97.51 ± 1.10	96.3 – 98.3
	5.0	99.83 ± 4.50	94.7 – 103.0
	10.0	100.48 ± 4.65	97.6 – 105.8
GSSG (*µg/ml*)	1.25	95.83 ± 3.61	93.8 – 100.0
	5.0	92.80 ± 14.48	81.7 – 109.2
	10.0	100.12 ± 2.59	98.2 – 103.1

CHAPTER 4: RESULTS AND DISCUSSION

This chapter provides a detailed description of the statistical methods used to analyse the data (Section 4.1), followed by the specific results of each of the analysed parameters (Section 4.2). In Section 4.3, these results are compared against available literature, and discussed against the background of the working hypothesis, stated in Chapter 1.

4.1 Statistical analyses

Statistical analyses were performed in IBM® SPSS® Statistics (version 28.0), assisted by Laerd Statistics® (https://statistics.laerd.com). All graphical representations were prepared in GraphPad Prism® (version 10) with the initial power analysis performed in G*Power (version 3; Universität Kiel, GER; Section 1.7). Effect magnitude calculations were done in Exploratory Software for Confidence Intervals (Cumming, 2013) and ® SPSS® Statistics.

All data sets were first screened for outliers (Grubbs' test with $\alpha = 0.05$ accepted as significant) and tested for normality of distribution and homogeneity of variances, with the Shapiro-Wilk and Levene's tests, respectively (only instances where data sets violated these assumptions are reported in figure or table legends). Regardless of the latter, independent *t*-tests with Welch correction (or Mann-Whitney *U*-test) were used for all analyses. A *p*-value < 0.05 (two-tailed) was accepted as significant, while $p \leq 0.07$ was considered an indication of a strong trend. All statistical analyses were however followed up with effect magnitude calculations (American Psychological Association, 2009; Cumming *et al.*, 2007) to support and inform statistical results. Specifically, the unbiased Cohen's *d* (d_{unb}) value (Cumming, 2013) was used (and reported with a 95 % confidence interval of the effect magnitude (du Sert *et al.*, 2020)), and considered large when $d_{unb} \geq 0.8$. To determine whether there were statistically significant sex differences for the parameters measured, an ordinary two-way ANOVA (analysis of variance) was performed, with sex and strain (or treatment) set as influencing factors. Here, only the main effect of sex is however reported to confirm visual inspection of sex distribution. For these analyses, the partial eta squared (η_p^2) was used as effect magnitude indicator, with $\eta_p^2 \geq 0.14$ (Ellis, 2010) accepted as a large effect. All descriptive statistics are reported as mean ± standard deviation, unless stated otherwise (indicated as footnotes).

4.2 Results

As summarized in Table 4-1, below, there were no statistically significant differences in terms of brain weight between the different experimental groups (*Strain effect*: $t_{25.0} = 1.27$, $p = 0.21$, d_{unb}

= 0.4 [-0.3; 1.1] and *FLX*[1] *effect*: $t_{30.0}$ = 0.71, p = 0.48, d_{unb} = 0.2 [-0.4; 0.9]). Importantly, because these samples were collected in another study (Oosthuizen, 2022), the pups were unfortunately not weighed prior to being euthanised and therefore, the brain weight cannot be expressed as a percentage of body weight. Regardless, these brain weights were used to calculate the concentrations of the various neurochemical markers, reported here.

Table 4-1: Descriptive statistics of the brain weight data of the different experimental groups
Whole brain weight of male and female FSL and FRL pups on PND22. ***95 % CI:*** *95 % confidence interval.* ***FRL:*** *Flinders resistant line.* ***FSL:*** *Flinders sensitive line.* ***PND:*** *Postnatal day.* ***SD:*** *Standard deviation.*

Strain	**Passively administered fluoxetine**	**Brain weight (*mg*) *mean ± SD (n)***	***95 % CI***	***Range***
FRL	***no***	993.7 ± 50.5 (*16*)	966.8 – 1021.0 mg	933.3 – 1086.0 mg
FSL	***no***	1024.0 ± 81.5 (*16*)	980.8 – 1068.0 mg	823.8 – 1158.0 mg
	yes	1045.0 ± 80.6 (*16*)	1002.0 – 1088.0 mg	911.0 – 1172.0 mg

4.2.1 Whole brain fluoxetine and norfluoxetine concentrations

Whole brain fluoxetine was undetectable (below the limit of detection) by the methods used in the current study. However, norfluoxetine, the active metabolite of fluoxetine, was successfully quantified, with passively administered fluoxetine during pre-pubertal development (from postnatal day 05 to 18) resulting in 41.28 ± 6.47 ng/g norfluoxetine in the whole brain of juvenile (PND21) FSL rats. Of note, this concentration was also comparable between male[2] and female pups ($t_{8.82}$ = 0.73, p = 0.48, d_{unb} = 0.4 [-0.6; 1.4]).

Moreover, when expressing the amount norfluoxetine to have reached the brain of pre-pubertal pups, as a percentage of the mean dose administered to the postpartum FSL dam, our findings suggest that 0.0014 ± 0.003 % of the mean daily fluoxetine dose[3] reached the brain of the FSL pups in the form of norfluoxetine.

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[1] *Fluoxetine*

[2] *One sample was excluded from the male group because of result being below the limit of detection.*

[3] *As described in Section 1.6, postpartum FSL dams received fluoxetine (10 mg/kg/day) via subcutaneous injections. Based on the data of Oosthuizen (2022), the average daily weight of these dams was 293.5 ± 21.1 g over the treatment period (i.e., PPD05 to 18). Using this daily average, the mean fluoxetine dose administered over the fourteen-day intervention period was 2.94 ± 0.21 mg/day, which was in turn used to calculate the percentage of norfluoxetine to have reached the offspring brain.*

4.2.2 Serotonin and 5-hydroxyindoleacetic acid

As summarized in Table 4-2, below, there were no statistically significant differences between FSL and FRL controls, regardless of sex, for either serotonin ($t_{27.7}$ = 0.473, p = 0.64, d_{unb} = 0.2 [-0.5; 0.9]) or 5-hydroxyindoleacetic acid ($t_{23.8}$ = 1.23, p = 0.23, d_{unb} = 0.3 [-0.3; 1.1]). Similarly, passively administered fluoxetine did not alter whole brain serotonin ($t_{27.1}$ = 0.48, p = 0.53, d_{unb} = 0.2 [-0.5; 0.9]) or its metabolite ($t_{20.0}$ = 0.11, p = 0.92, d_{unb} = 0.04 [-0.7; 0.7]) of FSL rats (irrespective of sex).

In support of the above-mentioned results, sex had no statistically significant impact on whole brain serotonin ($F_{1,\,28}$ = 0.23, p = 0.42, η_p^2 = 0.02) and 5-hydroxyindoleacetic acid ($F_{1,\,28}$ = 0.05, p = 0.82, η_p^2 = 0.002) in FSL and FRL rats. Similarly, passively administered fluoxetine had similar effects on whole brain serotonin ($F_{1,\,28}$ = 0.09, p = 0.77, η_p^2 = 0.003) and its metabolite ($F_{1,\,28}$ = 0.02, p = 0.90, η_p^2 = 0.001) in male and female FSL rats.

Table 4-2: Summary of serotonergic markers in the whole brain of FSL and FRL juvenile rats
The 5-HT and 5-HIAA values were used to calculate the 5-HIAA/5-HT ratio, reported in the text. ***5-HIAA:*** *5-hydroxyindoleacetic acid.* ***5-HT:*** *5-hydroxytryptamine.* ***FRL:*** *Flinders resistant line.* ***FSL:*** *Flinders sensitive line.* ***SD:*** *Standard deviation.*

Strain	Passively administered fluoxetine	5-HIAA (*ng/g*) *mean ± SD (n)*	5-HT (*ng/g*) *mean ± SD (n)*	5-HIAA/5-HT *mean ± SD (n)*
FRL	***no***	105.2 ± 16.4 (*16*)	33.28 ± 5.40 (*16*)	3.22 ± 0.64 (*16*)
FSL	***no***	95.06 ± 28.81 (*16*)	34.07 ± 4.01 (*16*)	2.80 ± 0.82 (*16*)
	yes	95.88 ± 11.90 (*16*)	34.67 ± 2.86 (*16*)	2.77 ± 0.27 (*16*)

As before, sex had no statistically significant influence on the serotonin turnover, either between FSL and FRL pups ($F_{1,\,28}$ = 0.09, p = 0.77, η_p^2 = 0.003), or between FLX and CRL FSL offspring ($F_{1,\,28}$ = 0.08, p = 0.78, η_p^2 = 0.003).

Serotonin turnover (5-HIAA/5-HT) was also comparable between strains (Figure 4-1; $t_{28.3}$ = 1.61, p = 0.12, d_{unb} = 0.6 [-0.1; 1.3]), with no statistically significant influence of passively administered fluoxetine in the whole brain of juvenile FSL rats (Figure 4-1; $t_{18.2}$ = 0.18, p = 0.86, d_{unb} = 0.06 [-0.6; 0.8]). Interestingly, the coefficient of variance for the pups that received fluoxetine via breast milk was lower than those that did not (9.74 % *vs*. 29.3 %).

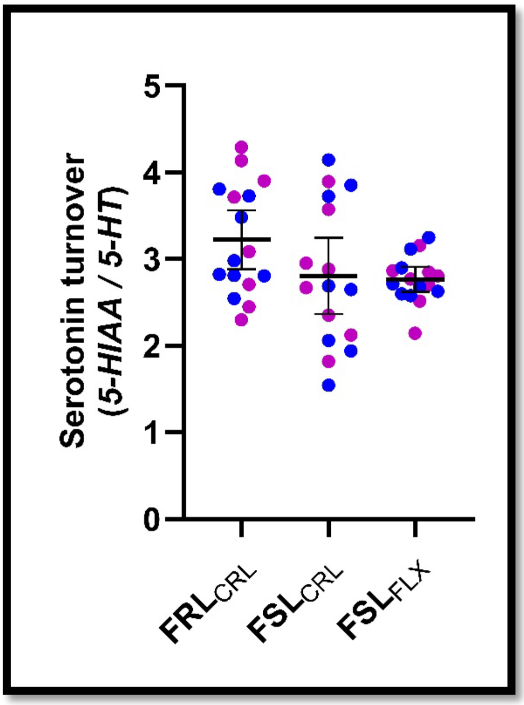

Figure 4-1: Whole brain serotonin turnover of FSL and FRL rats
Data points represent the mean ± 95% CI, with male and female indicated in blue and purple, respectively. Statistical results are reported in the text. ***5-HIAA:*** *5-hydroxyindoleacetic acid.* ***5-HT:*** *5-hydroxytryptamine.* ***CRL:*** *Control (distilled water).* ***FLX:*** *Fluoxetine (passively administered via breast milk).* ***FRL:*** *Flinders resistant line.* ***FSL:*** *Flinders sensitive line.*

4.2.3 Redox markers

As summarized in Table 4-3, there were statistically significant differences between FSL and FRL controls, regardless of sex, for both GSH ($U = 54$, $z = -2.79$, $p = 0.004$, $d_{unb} = 1.0$ [0.2; 1.7]) and GSSG ($U = 234$, $z = 4.00$, $p \leq 0.0005$, $d_{unb} = 1.8$ [1.0; 2.7]). In contrast, passively administered fluoxetine decreased whole brain GSH ($U = 187$, $z = -2.9$, $p = 0.003$, $d_{unb} = 1.8$ [1.0; 2.7]), without affecting GSSG concentrations ($U = 283$, $z = 0.72$, $p = 0.49$, $d_{unb} = 0.4$ [-0.3; 1.1]).

There were, however, no sex differences between FSL and FRL rats for either whole brain GSH ($F_{1,\ 28} = 2.31$, $p = 0.14$, $\eta_p^2 = 0.08$) or GSSG ($F_{1,\ 28} = 1.87$, $p = 0.18$, $\eta_p^2 = 0.06$) concentrations. Similarly, there were no statistically significant sex differences for FSL rats exposed to passively administered fluoxetine (*GSH*: $F_{1,\ 28} = 1.55$, $p = 0.22$, $\eta_p^2 = 0.05$; and *GSSG*: $F_{1,\ 28} = 1.69$, $p = 0.20$, $\eta_p^2 = 0.06$).

Table 4-3: Summary of redox markers in the whole brain of FSL and FRL juvenile rats
The GSH and GSSG values were used to calculate the GSH/GSSG ratio, reported in the text. ***FRL:*** *Flinders resistant line.* *a) Outlier identified but not removed from analysis.* *b) Data not normally distributed.* *c) When excluding the identified outlier, the fluoxetine effect narrowly misses statistical significance ($t_{28.6}$ = 2.03, p = 0.05, d_{unb} = 0.7 [-0.008; 1.4]).* ***FSL:*** *Flinders sensitive line.* ***GSH:*** *Glutathione.* ***GSSG:*** *Glutathione disulphide.* ***SD:*** *Standard deviation.*

Strain	Passively administered fluoxetine	GSH (*µg/g*) *mean ± SD (n)*	GSSG (*µg/g*) *mean ± SD (n)*	GSH/GSSG *mean ± SD (n)*
FRL	***no***	32.62 ± 7.07 (*16*)	163.3 ± 77.44 (*16*)	0.25 ± 0.12 (*16*)
FSL	***no***	25.54 ± 5.92 (*16*)	338.8 ± 106.7 (*16*)	0.08 ± 0.04 (*16*[a,b,c])
	yes	18.22 ± 6.75 (*16*)	384.1 ± 141.0 (*16*)	0.05 ± 0.03 (*16*)

The GSH/GSSG ratio was higher in juvenile FRL rats, compared to age matched FSL controls (Figure 4-2; U = 24, z = -3.92, $p \leq 0.0005$, d_{unb} = 1.8 [1.0; 2.7]). Interestingly, passively administered fluoxetine further decreased the GSH/GSSG ratio in juvenile FSL rats (Figure 4-2; U = 74, z = -2.04, p = 0.04, d_{unb} = 0.8 [0.05; 1.5]). Again, these findings were independent of sex (*Strain*: $F_{1,28}$ = 1.57, p = 0.22, η_p^2 = 0.05; and *Treatment*: $F_{1,28}$ = 0.25, p = 0.08, η_p^2 = 0.11).

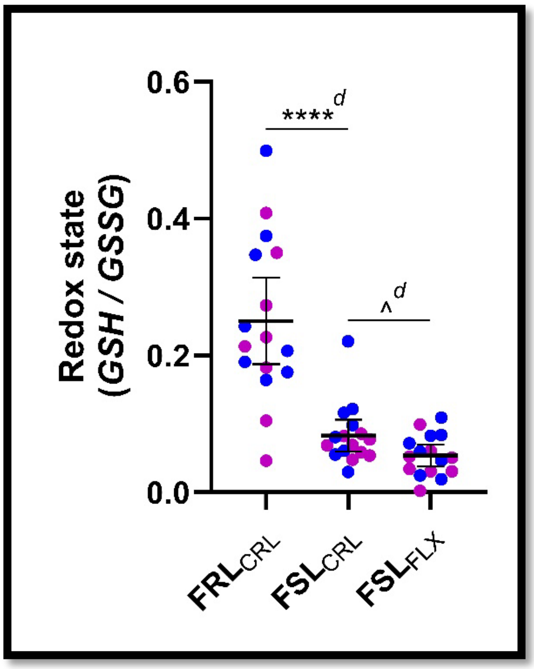

Figure 4-2: Whole brain redox status of FSL and FRL rats
*Data points represent the mean ± 95% CI, with male and female indicated in blue and purple, respectively. Statistical results are reported in the text, with **** p ≤ 0.0005, ^ p < 0.05, and d ≥ 0.8 (significant large effect) vs. indicated group.* ***GSH:*** *Glutathione.* ***GSSG:*** *glutathione disulphide.* ***CRL:*** *Control (distilled water).* ***FLX:*** *Fluoxetine (passively administered via breast milk).* ***FRL:*** *Flinders resistant line.* ***FSL:*** *Flinders sensitive line.*

4.3 Discussion

4.3.1 Model validation (FSL *vs.* FRL)

As described earlier (Section 2.6), the FSL rat is an approved, accepted, and validated model of depression. However, evidence for the depressive-like phenotype of this strain is mainly based on observations during adulthood, with less information available during the juvenile developmental period. The Malkesman group is leading the neuro-behavioural field in this regard, reporting on the usefulness of the FSL rat as a suitable model for childhood depression. Summarized, hypocortisolism (Braw *et al.*, 2006; Malkesman *et al.*, 2006), monoaminergic deficiencies (Malkesman *et al.*, 2008; Malkesman *et al.*, 2007; Whitney *et al.*, 2023b) and altered hippocampal redox status (Whitney *et al.*, 2023b), together with reduced brain (Whitney *et al.*, 2023b) and body weight (Malkesman *et al.*, 2006) have all been reported in juvenile FSL rats. These neurochemical differences were observed in the presence of increased behavioural despair (Whitney *et al.*, 2023b) and altered social behaviour. Interestingly, the increased social behaviour, displayed by these animals, could be a sign of aggression (Malkesman *et al.*, 2006), especially considering the association between antisocial behaviour and low cortisol levels observed in depressed children (Miller *et al.*, 2007). Finally, pre-pubertal FSL rats do not display signs of increased anxiety-like behaviour (Whitney *et al.*, 2023b) or anhedonia (Malkesman *et al.*, 2005), which is in line with the original description of the FSL rat as "*a model of depression without comorbid anxiety*" (Overstreet & Wegener, 2013).

Within this context, the current findings support the validity of the FSL rat as a suitable model for childhood depression (*see results reported in Section 4.2*). Although we previously reported increased serotonin turnover in PND38 male and female FSL rats (Whitney *et al.*, 2023b), the current findings identified no strain differences (Figure 4-1). This discrepancy could possibly be explained by the different brain areas analysed (hippocampus *vs.* whole brain), which prospective studies must consider. Still, Malkesman and colleagues (2007) also observed no strain differences between FSL and Sprague-Dawley juvenile controls in terms of serotonin turnover in the nucleus accumbens, yet did identify age-dependent differences. It could therefore be that monoaminergic changes occur over time that may (or may not) influence the behavioural profile of these animals. This hypothesis deserves further investigation, as the exact role of serotonin in the pathophysiology of depression has been questioned (Andrews *et al.*, 2015) and recently become a topic of debate (Jauhar *et al.*, 2023; Moncrieff *et al.*, 2022). Studies comparing the neurodevelopmental depressive-like phenotype of the Flinders line strain would be valuable to not only confirm the mentioned neurochemical differences, but also determine whether depressive-like behaviour changes over time.

Glutathione (GSH) is a potent intra- and extracellular antioxidant that when oxidised to GSSG (glutathione disulphide), reduces antioxidant protection. The concentration of GSH, relative to GSSG (GSH/GSSG) is therefore a valuable biomarker of cellular redox state, with lower levels indicating decreased antioxidant defences and consequently infers increased oxidative stress (Zitka *et al.*, 2012). In line with our previous findings (Whitney *et al.*, 2023b), the current results suggest that pre-pubertal FSL rats have reduced antioxidant defences (Figure 4-2), which may have contributed to the earlier mentioned, brain atrophy. This is valuable, as the immune-inflammatory and nitro-oxidative pathway is activated in patients with suicidal ideation and attempts (Vasupanrajit *et al.*, 2022), and correlates with our previous findings, suggesting PND21 male and female FSL rats to display psychomotor agitation (Whitney *et al.*, 2023b) – a characteristic of childhood depression (American Psychiatric Association, 2013b).

Our findings support the neurochemical construct of the FSL rat as a suitable model for childhood depression, presenting with decreased whole brain antioxidant defences. Further studies are however needed to validate the face validity (behavioural deficits) of this strain at such a young age. Based on these findings, the FSL rat is a suitable model to investigate the neurochemical effects of passively administered fluoxetine in the developing brain, despite having comparable whole brain serotonin concentrations on PND21.

4.3.2 The fluoxetine effect

Being a serotonergic-enhancing drug, fluoxetine could potentially alter serotonergic function in the developing brain of offspring exposed to it via breast milk. This is supported by the clinical response to serotonergic (and not noradrenergic) enhancing antidepressants in children and adolescents (Cipriani *et al.*, 2016; Murrin *et al.*, 2007). Moreover, that the serotonergic pathway matures before the adrenergic one, could further sensitize the developing brain to the effects of increased serotonin (Andersen, 2003; Andersen & Navalta, 2011). Consequently, we investigated the effects of passively administered fluoxetine in pre-pubertal FSL rats, specifically in terms of serotonin and oxidative stress, reported to be altered or compromised during juvenile development (Malkesman *et al.*, 2007; Whitney *et al.*, 2023b).

First, we were able to quantify whole brain fluoxetine and norfluoxetine levels, which to the best of our knowledge is novel. To this end, fluoxetine was undetectable, whilst the mean norfluoxetine concentration was quantified as 41.28 ± 6.47 ng/g in male and female FSL pups (Section 4.2.1). Our findings are supported by others (Epperson *et al.*, 2003; Heikkinen *et al.*, 2003; Hendrick *et al.*, 2001) who observed increased plasma norfluoxetine/fluoxetine ratios in infants exposed to passively administered fluoxetine. The translatability of our findings are further emphasized by Caccia and colleagues (1990) who investigated the pharmacokinetics of a single fluoxetine dose to a rat, and found that at lower doses (5 to 10 mg/kg; the latter being administered to the lactating dams of the current study), the kinetic profile of fluoxetine is generally comparable between rats and humans. Still, the species-specific nuances that must be noted is that the elimination time of fluoxetine and norfluoxetine is shorter in rats, than in humans (FLX: 4 to 7 h *vs.* 1 to 4 days; nFLX: 15 h *vs.* 7 days), and fluoxetine may follow non-linear kinetics from 10 mg/kg, and higher. In rats, peak plasma concentration of a single oral fluoxetine dose (5 and 10 mg/kg) is reached after 3.3 h (ranging between 1 and 6 h), with the volume of distribution and protein binding potential being similar between rats and humans (Caccia *et al.*, 1990). Furthermore, the mean whole brain concentrations of the parent compound (fluoxetine) and its metabolite (norfluoxetine), after a single 10 mg/kg oral dose at 3 h, were 2.4 ± 0.5 and 2.5 ± 0.5 nmol/g, with no preference for concentrating in the different brain regions. After 30 h, these levels decreased to 0.02 ± 0.01 and 1.1 ± 0.5 nmol/g, whilst the norfluoxetine/fluoxetine ratio increased from 1.0 to 54 (Caccia *et al.*, 1990). Although not quantified here, available data (Berle *et al.*, 2004; Heikkinen *et al.*, 2003; Kim *et al.*, 2006; Oberlander *et al.*, 2005; Taddio *et al.*, 1996; Weissman *et al.*, 2004) suggests that fluoxetine is indeed expressed in the breast milk at a mean 3.96 ± 2.25[1] % of the maternal weight-adjusted dose. It would therefore follow that, because of the four-day wash-out period between the final dose administered to the postpartum dams (used for the current study), the dose to which the pups were exposed may have significantly decreased. Also, although rodents are generally weaned on PND21, it may be that some of them are already dependent on solid food (i.e., rat chow supplied) at this age, and therefore consuming less (if any) breast milk (and fluoxetine). Either way, this could explain why we were unable to detect fluoxetine in the juvenile brains. Follow-up studies should therefore measure fluoxetine concentrations immediately after the final maternal dose, to confirm our findings. The prolonged half-life of norfluoxetine most probably contributed to the detectable brain concentrations. And that norfluoxetine shares the serotonergic-enhancing potency of fluoxetine (Heikkinen *et al.*, 2003), could alter serotonin levels in the pre-pubertal brain.

In this regard, whole brain serotonin, its metabolite (5-hydroxyindoleacetic acid) and serotonin turnover of pre-pubertal male and female FSL rats, was unaffected by passively administered fluoxetine (Figure 4-1 and Table 4-2). Although unexpected, this is supported by the work of Epperson and colleagues (2003) who reported comparable baseline and post-exposure plasma serotonin levels in the babies of breastfeeding mothers treated with fluoxetine. More recently,

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[1] *Mean ± standard deviation.*

de Andrade Silva and colleagues (2023), reported that 21-days of 10 mg/kg of sub-cutaneous fluoxetine did not induce any serotonin transporter or receptor expression differences in the hippocampus of juvenile Wistar rats. These, together with the current findings, support the results of Zaccarelli-Magalhães (2020), reporting no behavioural changes observed in Wistar offspring, exposed to escalating doses of passively administered fluoxetine.

As for the effect on redox state, we observed that passively administered fluoxetine further compromised whole antioxidant defences in male and female FSL pups, as indicated by decreased GSH (Table 4-3) and a GSH/GSSG ratio (Figure 4-2). It must however be noted here that these redox state differences are dependent on one data point, identified as a statistical outlier. However, because this specific sample had no obvious anatomical defect, nor was it identified as an outlier in the serotonergic analyses, it was included in the analysis. To promote transparency, even when excluded, a strong trend for this fluoxetine effect remains (Table 4-3), validating our findings and interpretation thereof. Regardless, our findings are similar to that of de Andrade Silva and colleagues (2023), who observed decreased hippocampal GSH/GSSG ratios, as well as decreased glutathione *S*-transferase (a family of enzymes, involved in reducing oxidative stress by conjugating GSH and forming non-toxic products, which has been shown to be reduced in depressed patients (Gawryluk *et al.*, 2011)). What is however interesting is that hippocampal malondialdehyde, superoxide dismutase and BDNF were unaffected, whereas the NAD/NADH[1] ratio was increased in these pups – altogether suggesting decreased oxidative stress damage, and improved mitochondrial function (de Andrade Silva *et al.*, 2023). These are interesting observations, as Dalmizrak and colleagues (2019) have argued that although fluoxetine is known to decrease glutathione reductase (the enzyme responsible for regulating glutathione levels), and consequently decrease antioxidant defences and increase oxidative stress, this effect could be beneficial in combating intracellular abnormalities, such as tumour cells. That no evidence of increased oxidative stress damage (i.e., unaltered malondialdehyde and BDNF levels) were observed in a similar investigation (de Andrade Silva *et al.*, 2023), could either be explained by compensatory mitochondrial mechanisms (i.e., increased NAD/NADH), and/or by the mitochondrial altering effects, induced by fluoxetine (Emmerzaal *et al.*, 2021). Either way, these observations point towards a more complicated redox state effect of passively administered fluoxetine that warrants further investigation. Moreover, whether these neurochemical effects would benefit or impair behaviour in these juvenile animals, remains unclear and must also be investigated in prospective studies. Finally, although passively administered fluoxetine appears to decrease

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[1] *Nicotinamide adenine dinucleotide in its oxidized (NAD^+) and reduced (NADH) forms.*

the antioxidant defences, direct markers of oxidative stress damage, such as malondialdehyde or even 8-hydroxy-2'-deoxyguanosine, must be measured in prospective studies to conclude whether the reported neurochemical effects are indeed deleterious.

CHAPTER 5: CONCLUSION, LIMITATIONS, AND FUTURE RESEARCH RECOMMENDATIONS

Based on the results and discussion of Chapter 4, this chapter addresses each of the research questions outlined in Chapter 1. These answers are then used to formulate a general conclusion and response to the overarching research question, posed in Section 1.3. Following this conclusion, the shortcomings and limitations of the current study are highlighted and used to guide further studies into this research topic by suggesting methodology to be investigated.

Study question one

Are there neurochemical differences in juvenile (PND21) FSL rats, compared to FRL controls?

Yes. Although only serotonin and markers of redox state were measured, the current findings highlighted strain differences in terms of the latter. These findings suggest a compromised antioxidant defence system in the brain of pre-pubertal FSL rats, that may contribute to brain atrophy (Whitney *et al.*, 2023b) and the depressive-like phenotype reported by others. In terms of the current report, the null hypothesis (Section 1.5), can therefore, respectively be accepted (for serotonin) and rejected (for redox state).

Study question two

Can the concentration of passively administered fluoxetine to reach the juvenile brain be quantified? If indeed, what is this concentration?

Yes. Although whole brain fluoxetine levels were below the limit of detection, we were able to successfully quantify norfluoxetine in the whole brains of pre-pubertal male and female FSL rats. Based on the current findings, passively administered fluoxetine resulted in an average norfluoxetine concentration of 41.28 ± 6.47 ng/g, which translates to 0.0014 ± 0.003 % of the mean daily fluoxetine dose administered to the lactating dams during the early postpartum period and following a four-day wash-out period.

Study question three

What are the effects of passively administered fluoxetine on serotonin levels and markers associated with mitochondrial function and oxidative stress?

Based on the current findings, passively administered fluoxetine had no effect on whole brain serotonin levels, or the metabolism thereof. In contrast, passively administered fluoxetine appears to further decrease antioxidant defences, compared to treatment naïve controls. As to whether this decrease is detrimental to the depressive-like phenotype of the juvenile FSL rat, remains unknown, and requires further investigation. Based on these findings (including those related to Study question 1), we can accept the null hypothesis that passively administered fluoxetine will not reverse the redox state deficit of pre-pubertal male and female FSL rats.

Study question four

Does sex influence the outcomes of research questions one, two and three?

No. There were no obvious or statistically significant sex differences in any of the parameter, measured in the current study. This could be explained by the fact that the pups were pre-pubertal when hormonal influences are non-significant. We can therefore accept the null hypothesis for this specific study question.

5.1 Conclusion

In this research work, we were able to quantify whole brain norfluoxetine concentrations of passively administrated fluoxetine and evaluate the serotonergic and redox state effect in the developing brain of a pre-pubertal rodent model of depression. Our findings confirm that passively administered fluoxetine does reach the infant brain in the form of norfluoxetine, which may manipulate processes of oxidative stress regulation. As to the nature of this manipulation, further studies are required to determine whether the observed decrease in antioxidant defences adversely influence juvenile behaviour. Taken together, further studies into the long-term bio-behavioural effects are needed to effectively inform breastfeeding mothers on the safety of antidepressant-use during the postpartum period.

5.2 Limitations and future recommendations

Several limitations were identified in the current study together with questions that should addressed in future studies. These are briefly described and elaborated on, below:

Limitation 1

Including broader neurochemical profile analyses. Because the current study only investigated the serotonergic and oxidative stress effects of passively administered fluoxetine, prospective studies should also include other markers that potentially could have been affected. To this end, brain-derived neurotrophic fact (BDNF), norepinephrine and other markers related to oxidative stress (i.e., malondialdehyde and/or 8-hydroxy-2'-deoxyguanosine) and mitochondrial function (complex respiration and/or NAD/NADH[1]) would be valuable considerations. Although the serotonergic pathways mature earlier than the adrenergic ones, neurochemical imbalances caused by early-life insults could offset the developmental trajectory and function of the noradrenergic system, leading to bio-behavioural deficits. The effects of passively administered fluoxetine on the antioxidant defences also require further investigation, to determine whether the hypothesized mitochondrial compensatory mechanisms are indeed induced.

Limitation 2

Including behavioural analyses. Although not practical in the current study, prospective studies should investigate the behavioural effects of passively administered fluoxetine in the FSL rats, to be correlated with the mentioned neurochemical analyses. Potential behavioural analyses could include the forced swim test (behavioural despair), novel object recognition test (cognitive function), open field test (general locomotor activity and anxiety-like behaviour), sucrose preference test (anhedonia), and the novelty suppressed feeding test (anxiety and motivation) to better understand the impact of passively administered fluoxetine on offspring development.

Limitation 3

Investigating the developmental stress-sensitivity of FSL rats exposed to passively administered fluoxetine. Linking to the above-mentioned shortcoming of not being able to include behavioural analyses, future studies must also consider analysing the bio-behavioural profile of FSL rats, exposed to passively administered fluoxetine, at different developmental ages. Because children being exposed to passively administered fluoxetine are assumed to have a greater risk for developing depression (because of the genetic risk factor, and an assumed accurate maternal depression diagnosis), it would be valuable to determine whether these children may also be more sensitive to external stressors. To this end, follow-up studies could also include an external stressor, such as maternal or social isolation, to determine whether the passively exposed offspring, present with any bio-behavioural evidence of an altered stress-response and sensitivity.

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[1] *Nicotinamide adenine dinucleotide in its oxidized (NAD^+) and reduced (NADH) forms.*

ADDENDUM A: ETHICAL APPROVAL

Ethical approval was both granted (NWU) and waived (Wits) by the necessary Institutions – the certificates are presented below. For context, because the neurochemical analyses were performed at the North-West University, ethical approval had to be gained from the AnimCare-REC Committee, whilst ethical approval (or waiver) was also required from University of the Witwatersrand, to whom the degree belongs to. As alluded to in Chapter 1, ethical approval for the use of these samples is covered by application NWU-00434-21-A5.

A1 Ethical approval by North-West University

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

10 August 2023

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 10/08/2023, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Determining brain concentrations of passively administered fluoxetine in Flinders sensitive line rat offspring and the neurochemical effects thereof
Principal Investigator/Study Supervisor/Researcher: Dr SF Steyn
Student: -

Ethics number: N W U - 0 0 7 8 9 - 2 3 - A 5
Institution Study Number Year Status
Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 10/08/2023 **Risk:** Category 0
Expiry date: 31/08/2024

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of August annually until completion of the study.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*

9.1.5.4.2 Ethics Approval Letter of Study 1

- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
    - *request access to any information or data at any time during the course or after completion of the study;*
    - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
    - *withdraw or postpone approval if:*
        - *any unethical principles or practices of the study are revealed or suspected;*
        - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
        - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
        - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by
Christiaan B Brink
(Tiaan)
Date: 2023.08.11
14:25:56 +02'00'

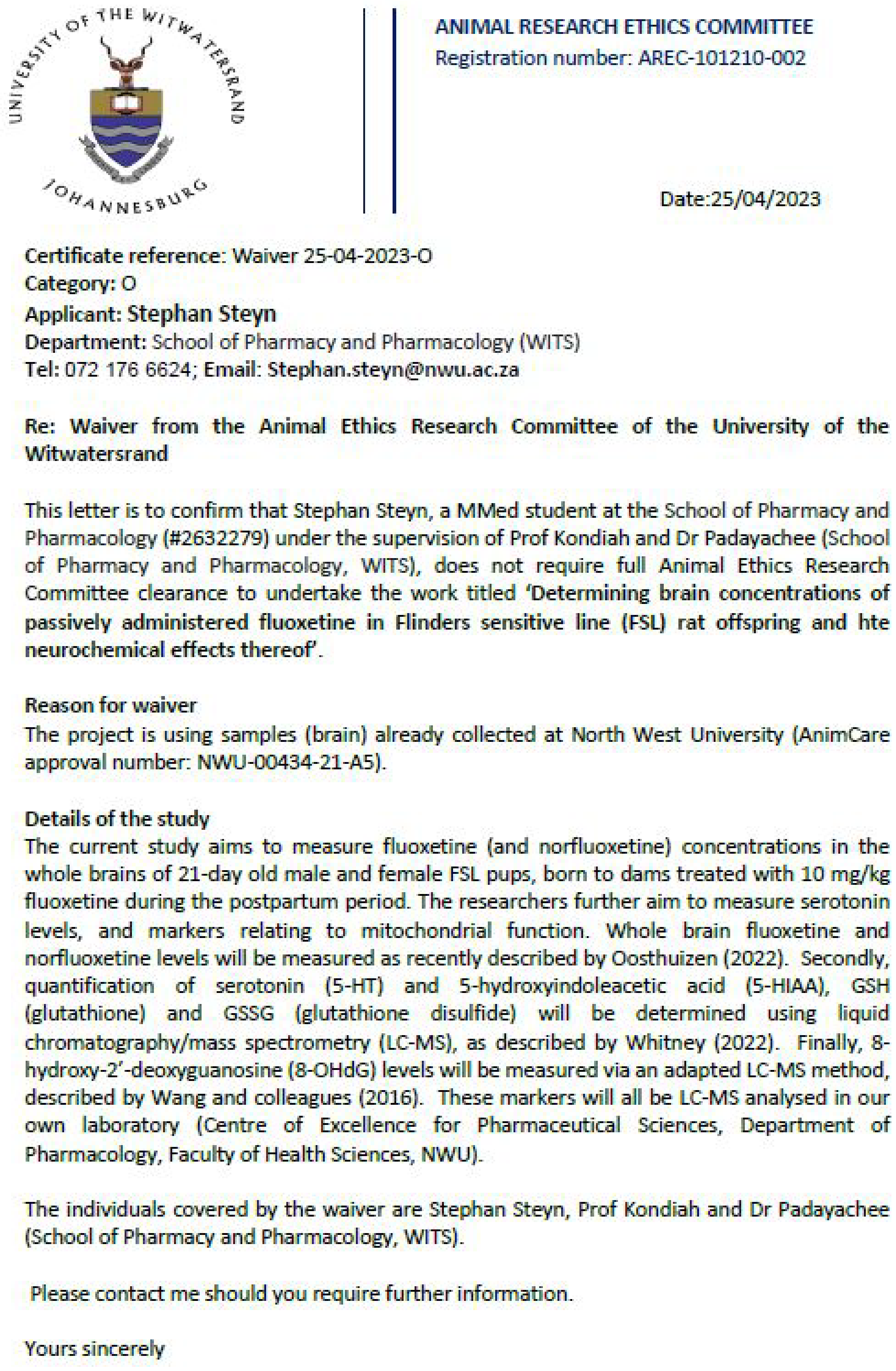
Chairperson: NWU-AnimCareREC

Current details: (22654704) G:\One Drive\NWU-AnimCareREC\9.1.5.1_NWU-AnimCareREC Ethics\NWU-00789-23-A5 \9.1.5.1.1_FV\9.1.5.4_Approval

File Reference: 9.1.5.4

9.1.5.4.2 Ethics Approval Letter of Study 2

A2 Ethical waiver by the University of the Witwatersrand



ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date:25/04/2023

Certificate reference: Waiver 25-04-2023-O
Category: O
Applicant: Stephan Steyn
Department: School of Pharmacy and Pharmacology (WITS)
Tel: 072 176 6624; **Email: Stephan.steyn@nwu.ac.za**

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Stephan Steyn, a MMed student at the School of Pharmacy and Pharmacology (#2632279) under the supervision of Prof Kondiah and Dr Padayachee (School of Pharmacy and Pharmacology, WITS), does not require full Animal Ethics Research Committee clearance to undertake the work titled **'Determining brain concentrations of passively administered fluoxetine in Flinders sensitive line (FSL) rat offspring and hte neurochemical effects thereof'**.

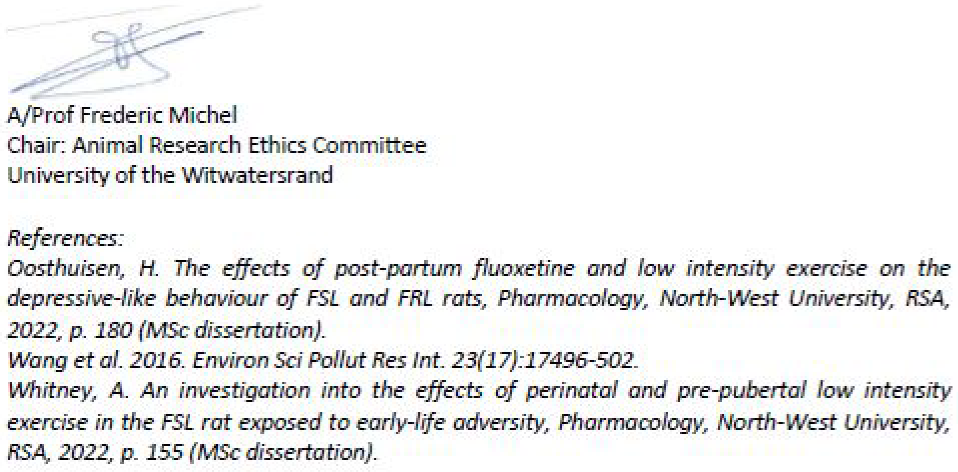
Reason for waiver
The project is using samples (brain) already collected at North West University (AnimCare approval number: NWU-00434-21-A5).

Details of the study
The current study aims to measure fluoxetine (and norfluoxetine) concentrations in the whole brains of 21-day old male and female FSL pups, born to dams treated with 10 mg/kg fluoxetine during the postpartum period. The researchers further aim to measure serotonin levels, and markers relating to mitochondrial function. Whole brain fluoxetine and norfluoxetine levels will be measured as recently described by Oosthuizen (2022). Secondly, quantification of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), GSH (glutathione) and GSSG (glutathione disulfide) will be determined using liquid chromatography/mass spectrometry (LC-MS), as described by Whitney (2022). Finally, 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels will be measured via an adapted LC-MS method, described by Wang and colleagues (2016). These markers will all be LC-MS analysed in our own laboratory (Centre of Excellence for Pharmaceutical Sciences, Department of Pharmacology, Faculty of Health Sciences, NWU).

The individuals covered by the waiver are Stephan Steyn, Prof Kondiah and Dr Padayachee (School of Pharmacy and Pharmacology, WITS).

Please contact me should you require further information.

Yours sincerely



A/Prof Frederic Michel
Chair: Animal Research Ethics Committee
University of the Witwatersrand

References:

Oosthuisen, H. The effects of post-partum fluoxetine and low intensity exercise on the depressive-like behaviour of FSL and FRL rats, Pharmacology, North-West University, RSA, 2022, p. 180 (MSc dissertation).

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Whitney, A. An investigation into the effects of perinatal and pre-pubertal low intensity exercise in the FSL rat exposed to early-life adversity, Pharmacology, North-West University, RSA, 2022, p. 155 (MSc dissertation).

A3 Research proposal approval certificate

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Private Bag 3 Wits, 2050
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Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

21 September 2023
Person No: 2632279
PAG

Dr SF Steyn
Njala street
Njala Park
Baillie Park
2531
South Africa

Dear Dr Stephanus Steyn

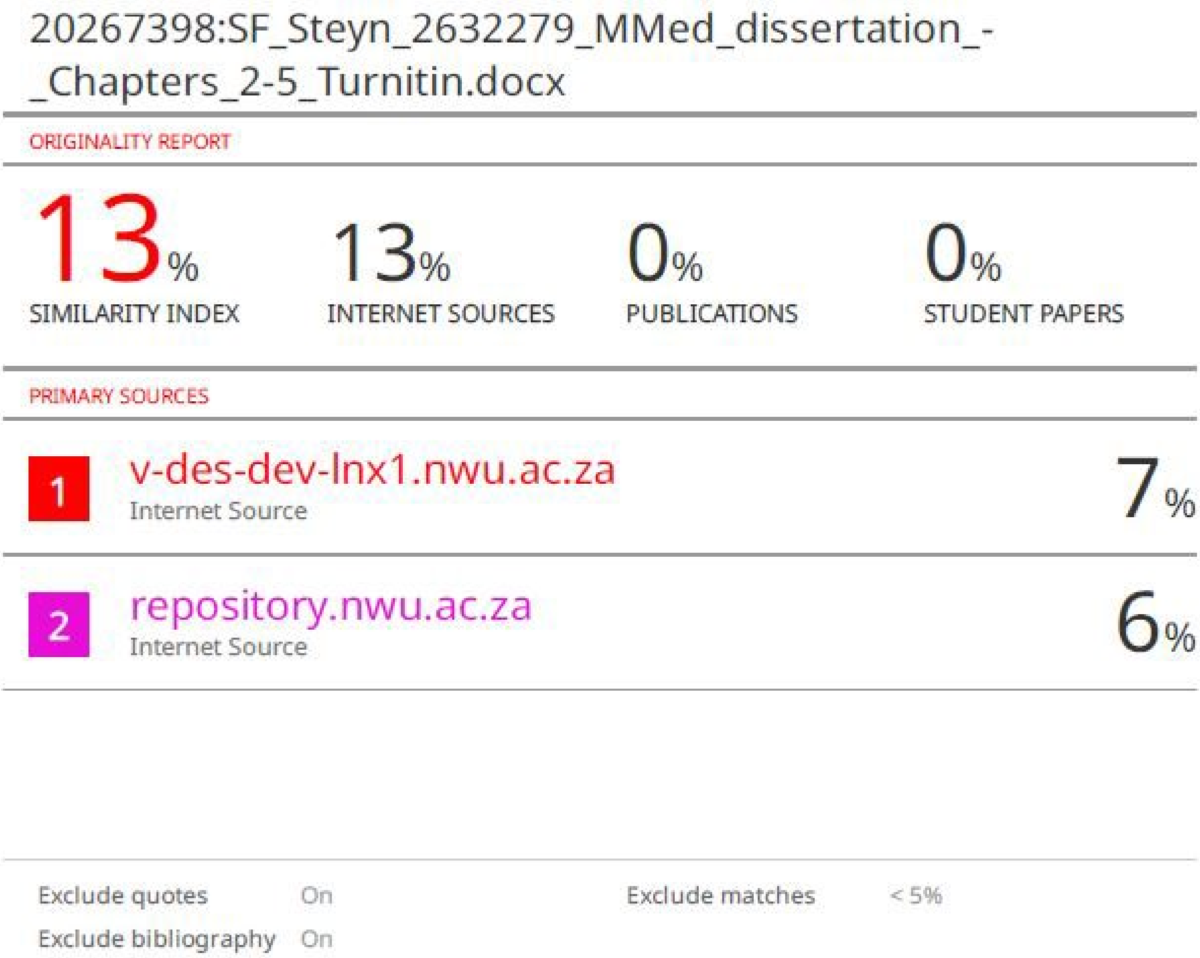
Master of Science in Medicine: Approval of Title

We have pleasure in advising that your proposal entitled *Brain concentrations and the neurochemical effects of passively administered fluoxetine in Flinders sensitive line rat offspring* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

ADDENDUM B: TURNITIN SUMMARY REPORT



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