# Development and validation of an animal model of treatment-resistant depression with psychotic features

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# DEDICATION

I dedicate this work to my Lord, the risen Saviour, Jesus Christ. This degree and the journey leading up to it are testament to His Grace, Power, Unfailing Love, and provision. I am ever grateful to the Most High God, El-Elyon, for carrying me this far. I am *convinced* not only that He who has begun a good work in me will continue to perfect *and* complete it (Phil 1:6, AMP) but that, indeed, "I CAN do ALL things through Christ who strengthens me" (Phil 4:13, NKJV).

**"…'Not by might, nor by power, but by My Spirit', says the Lord of Hosts."**

**– Zechariah 4:6 (NKJV)**

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# ABSTRACT

Major depression (MD) affects up to 17% of the global population. While effective treatments for MD exist, an underlying interaction between genetic propensity and chronic and/or early life adversity may render these drugs ineffective in managing the disorder. Treatment resistant depression (TRD) is a growing problem with various risk factors. Although its underlying biology remains elusive, comorbid psychosis is known to contribute to its development. MD with psychotic features, also known as psychotic depression (MDpsy) is one such disorder wherein the patient presents with co-occurring depressive and psychotic symptoms. MDpsy is characterised by depressive symptoms that are more severe than those occurring in MD, but similar to SCZ and bipolar disorder (BD), while presenting psychosis is generally less severe than in SCZ. This disorder is often misdiagnosed as MD or schizophrenia, resulting in patients not receiving appropriate treatment which further contributes to the development of treatment resistance. While antidepressant or antipsychotic monotherapy is ineffective, a combination of fluoxetine (FLX) and olanzapine (OLZ), originally approved by the United States Food and Drug Administration (FDA) for the treatment of TRD, has proven useful in treating MDpsy.

A lack of animal models of MDpsy has hindered research and development into new and improved treatments. Thus we sought to develop such a model based on a gene-X-environment approach to give insight into the pathophysiology of MDpsy as well as to provide a platform for the development of more effective treatment strategies. The Flinders Sensitive Line (FSL) is a genetic rodent model of MD, while post-weaning social isolation rearing (SIR) is a neurodevelopmental model of schizophrenia. Both models reflect the bio-behavioural features of their respective human disorder. We hypothesised that combining the two models would produce an animal that presents with the bio-behavioural features of MDpsy later in life. The current investigation was thus conducted to achieve the following three aims: 1) establish the depressive-like profile of the FSL-SIR rat and resistance to FLX; 2) establish the psychotic-like profile of the FSL-SIR rat and its resistance to or partial improvement with OLZ; 3) demonstrate therapeutic benefits across mood and psychotic manifestations in FSL-SIR rats following OLZ-FLX combination (OFC) treatment.

# Abstract

To establish the SIR-exposed FSL (FSL-SIR) rat as a model of TRD, male FSL rats were reared socially or in isolation for 8 weeks from post-natal day (PND) 21. These rats received either saline (1 mg/kg, SAL) or FLX (10 mg/kg) subcutaneously (s.c) for 14 days from PND 63. Coping and depressive-like behaviour, locomotor activity, recognition memory and social interactive behaviours were assessed in the forced swim test (FST), open field test (OFT), novel object recognition test (nORT), and social interaction test (SIT), respectively. To establish the FSL-SIR as a model of MDpsy, male SD and FSL rats were reared in isolation for 8 weeks from PND 21. These rats received either saline (1 mg/kg, SAL) or OLZ (5 mg/kg) subcutaneously (s.c) for 14 days from PND 63. To assess the therapeutic effects of OFC in the FSL-SIR rats, another group of FSL-SIR rats received a combination of FLX and OLZ (OFC), at doses of 10 mg/kg and5 mg/kg, respectively. The effects of OLZ and OFC in the FST were also assessed in FSL-SIR rats. Sprague-Dawley (SD) rats receiving only SAL treatment were used in both studies as the healthy control. Post-mortem cortico-hippocampal monoamine levels were subsequently determined by high performance liquid chromatography (HPLC) while plasma dopamine-β-hydroxylase (DBH), corticosterone (CORT), interleukin-6 (IL-6), tumour necrosis factor (TNF-α), and brain-derived neurotrophic factor (BDNF) levels were determined by enzyme-linked immunosorbent assay (ELISA).

FSL displayed significant manifestations of depressive-like, behaviours, including, a trend to psychomotor retardation in the OFT, significantly elevated immobility (despair) and reduced swimming (survival behaviour) in the FST, and significant social withdrawal and social anxiety-like behaviour in the SIT versus SD-SAL rats. Significant reversals in these deficits were observed following FLX treatment while a trend toward reduced social anxiety-like behaviour was observed. Climbing behaviour in the FST was unaffected by condition or treatment in FSL rats and not altered by FLX. Downward trends in novelty discrimination were observed in the FSL rats and left unchanged by FLX. FSL rats presented with significantly reduced cortico-hippocampal NE and hippocampal 5-HT and frontocortical 5-HT and cortico-hippocampal DA levels similar to SD controls. FLX did not reverse these deficits but rather significantly reduced hippocampal 5-HT. Plasma DBH, CORT, IL-6, and TNF-α levels were unchanged in FSL versus SD rats. All except for CORT were unaltered by FLX. FLX treatment significantly increased plasma CORT in FSL rats. The downward trend in plasma BDNF noted in these rats was continued by FLX treatment. Locomotor activity was increased by a large effect size in SAL-treated FSL-SIR rats versus FSL-SAL rats while a trend toward reduced activity followed FLX treatment. Like socially-housed FSL rats, FSL-SIR rats

displayed significant immobility versus SD animals, although less than FSL rats by a large effect size. Swimming was similar in FSL-SIR rats and SD controls while there was an upward trend in climbing. FLX significantly increased immobility and decreased swimming versus SD rats. OLZ and OFC did not improve immobility but rather significantly reduced swimming in these rats. The upward trend in climbing was continued by FLX but reversed by OLZ and OFC. FSL-SIR did not display cognitive impairment in the nORT, however, FLX showed a trend to improve novelty discrimination. FSL-SIR rats exhibited significant disordered social behaviour and asocial (anxious) behaviour versus SD rats. Neither was reversed by FLX. Significant cortico-hippocampal NE and 5-HT were observed while downward trends in cortico-hippocampal DA were observed in FSL-SIR rats. FLX significantly reversed all these deficiencies. DBH was significantly reduced in FSL-SIR versus SD rats, with a very large effect size reduction versus SAL-treated FSL rats. There were not reversed by FLX. CORT was not significantly elevated in FSL-SIR versus FSL controls and remained unaltered by FLX. FSL-SIR rats did not present with significant elevations in IL-6 and TNF-α although a downward trend in TNF-α followed FLX treatment. Although presenting similarly to SD rats, BDNF in FSL-SIR rats followed a downward trend after treatment with FLX.

SD-SIR exhibited psychotic-like behaviour manifested as significantly decreased %PPI which was not improved by OLZ treatment. SD-SIR rats did not exhibit hyperlocomotion but did trend toward thigmotactic behaviour in the OFT versus SD controls which was not improved by OLZ treatment. SD-SIR rats did not manifest social withdrawal, anxiety or aggression in the SIT. SD-SIR rats presented with trends toward reduced cortico-hippocampal NE and raised cortico-hippocampal 5-HT levels versus SD rats were observed. These aberrations were normalised by OLZ treatment. Cortico-hippocampal DA was similar in SD-SIR and SD rats. Plasma DBH was significantly reduced in SD-SIR rats and trended toward a further reduction following OLZ treatment. An upward trend in SD-SIR plasma CORT levels versus SD controls was observed. This was reversed by OLZ treatment. Elevated plasma IL-6 and TNF-α levels were not observed in SD-SIR rats and both remained unaltered by OLZ treatment. FSL-SIR rats did not exhibit hyperlocomotion but did display significant thigmotactic behaviour with downward trends and significant reductions in both parameters following OLZ and OFC, respectively. While FSL-SIR did not exhibit cognitive impairment in the nORT, a trend to increase novelty discrimination was noted following treatment with OLZ. OFC had no effect on novelty discrimination. Significant social withdrawal and social anxiety-like behaviour were observed in the FSL-SIR versus SD controls; these were not improved

by OLZ or OFC treatment. There was a trend to aggression by the FSL-SIR rats which was attenuated by OLZ and normalised by OFC. Significantly reduced %PPI in the FSL-SIR rat was not improved by OLZ but a trend to increase %PPI followed OFC treatment. Significant cortico-hippocampal NE and 5-HT were observed while downward trends in cortico-hippocampal DA were observed in the FSL-SIR rat. OLZ and OFC caused a significant rise in cortico-hippocampal NE and 5-HT in FSL-SIR rats. Upward trends in cortico-hippocampal DA followed OLZ and OFC treatment. OLZ and OFC did not alter the trend toward raised CORT in the FSL-SIR versus SD rats. Neither OLZ nor OFC altered the downward trend in plasma DBH levels noted in the FSL-SIR rat. Although not significantly different from SD controls, OLZ and OFC showed a trend to reduce both plasma IL-6 and TNF-α. BDNF was not altered in the FSL-SIR rat; however, an upward trend in this neurotrophin followed OLZ treatment. OFC had no effect on BDNF in these rats.

These results confirm that exposing FSL rats to post-weaning SIR produces animals that present with both depressive- and psychotic-like behaviours akin to those observed in MDpsy, including immobility in the FST, decreased %PPI, psychomotor agitation, social impairment, anxiety, and aggression and accompanied by increased plasma CORT and reduced DBH. These depressive- and psychotic-like symptoms as well as CORT and DBH demonstrate resistance to FLX and OLZ monotherapy while %PPI was partially responsive to OFC.

Keywords: treatment resistant depression, psychotic depression, Flinders Sensitive Line rat, social isolation rearing, bipolar disorder, forced swim test, prepulse inhibition, social interaction test, fluoxetine/olanzapine, dopamine-β-hydroxylase

# ABBREVIATIONS

| | |
|---|---|
| 3-MT | 3-Methoxytyramine |
| 5-HIAA | 5-hydroxyindoleacetic acid |
| 5-HMT | 5-hydroxy-Nω-methyltryptamine oxalate |
| 5-HT | Serotonin |
| ACTH | Adrenocorticotrophic hormone |
| ANOVA | Analysis of variance(s) |
| APA | American Psychological Association |
| AREC | Animal Research Ethics Committee |
| BD | Bipolar disorder |
| BDNF | Brain-derived neurotrophic factor |
| COMT | Catechol-O-methyltransferase |
| CORT | Corticosterone |
| CSF | Cerebrospinal Fluid |
| DA | Dopamine |
| DAT | Dopamine transporters |
| DBH, DβH | Dopamine-β-hydroxylase |
| DOPAC | 3,4-dihydroxyphenylacetic acid |
| DSM-V | Diagnostic and Statistical Manual of Mental Disorders, fifth edition |
| DST | Dexamethasone Suppression Test |
| ECT | Electroconvulsive therapy |
| FC | Frontal cortex |
| FDA | Food and drug administration |
| FLX | Fluoxetine |
| FSL | Flinders Sensitive Line |
| FST | Forced swim test |
| GRIK4 | Glutamate Receptor Ionotropic Kainate 4 |
| GRIN2B | Glutamate N-methyl-D-aspartate receptor 2B subunit |
| GWAS | Genome-wide association studies |

# Abbreviations

| | |
|---|---|
| HC | Hippocampus |
| HDRS | Hamilton Depression Rating Scale |
| HPA-axis | Hypothalamic-pituitary-adrenal axis |
| IDO | Indoleamine 2,3-dioxygenase |
| IFNγ | Interferon gamma |
| IL | Interleukin |
| IL-1RA | IL-1 receptor antagonist |
| IL-6 | Interleukin-6 |
| JAD | Journal of Affective Disorders |
| kg | Kilogram(s) |
| MADRS | Montgomery–Åsberg Depression Rating Scale |
| MAOIs | Monoamine oxidase inhibitors |
| MD | Major depression; depression |
| MDE | Major depressive episode |
| MDpsy | Major depression with psychotic features; psychotic depression |
| mg | Milligram |
| MHPG | 3-methoxy-4-hydroxyphenylglycol |
| min | Minute(s) |
| mL | Millilitre(s) |
| $NAD^+$ | Nicotinamide adenine dinucleotide |
| NE | Norepinephrine |
| NET | Norepinephrine transporters |
| ng | Nanogram(s) |
| NHREC | National Health Research Ethics Council |
| NMDA | N-methyl-D-aspartate |
| NO | Nitric oxide |
| nORT | Novel object recognition test |
| NOS | Nitric oxide synthase |
| NRIs | NE re-uptake inhibitors |
| NWU | North-West University |
| OFC | Olanzapine+Fluoxetine combination |
| OFT | Open Field Test |

# Abbreviations

| | |
|---|---|
| OLZ | Olanzapine |
| PBD | Psychotic bipolar disorder |
| PCDDP | Pre-clinical Drug Development Platform |
| pg | Picogram(s) |
| PND | Post-natal day |
| PPI | Prepulse inhibition test |
| PTSD | Post-traumatic stress disorder |
| rACC | Rostral anterior cingulate cortex |
| rCBF | Regional cerebral blood flow |
| SAL | Saline |
| SAVC | South African Veterinary Council |
| SCZ | Schizophrenia |
| SD | Sprague-Dawley |
| SERT | Serotonin transporters |
| sgACC | Subgenual anterior cingulate cortex |
| sIL-6R | Soluble IL-6 receptor |
| SIR | Social isolation rearing |
| SIT | Social interaction test |
| SOC | Social rearing |
| SPECT | Single Photon Emission Computed Tomography |
| SSRI | Selective serotonin reuptake inhibitor |
| TDS | Time-dependent sensitisation |
| TNF-α | Tumour necrosis factor-alpha |
| TRD | Treatment resistant depression |
| WHO | World Health Organisation |

# CONTENTS

# Contents

# CHAPTER 1: INTRODUCTION

## 1. Thesis layout

This thesis is compiled in article format as prescribed and approved by the North-West University. The main findings of the study are presented in two research manuscripts for possible publication in international peer-reviewed journals, one (Manuscript A) is currently in review and the other (Manuscript B) in readiness for submission. Additional findings pertaining to neuroplastic anomalies as well as other relevant data pertaining to the respective manuscripts are presented as addenda.

### Chapter 1: Introduction

Chapter 1 provides a brief description of the problem statement, study questions, aims, a framework of the study layout, and expected outcomes.

### Chapter 2: Literature review

This chapter discusses the literature background supporting the study and from which the hypotheses and aims of the study are based. This literature review provides more in-depth background of the studies presented in Manuscripts A and B.

### Chapter 3 and 4: Manuscripts prepared for peer-reviewed journals

The manuscripts are presented in the format prescribed by the NWU for submission for thesis examination.

**Chapter 3** presents the findings regarding development of a rodent model of treatment-resistant depression that exhibits depressive-like behaviour resistant to fluoxetine. This manuscript (**Manuscript A)** is in review by the *Journal of Affective Disorders* (Elsevier).

**Chapter 4** presents the findings regarding the psychotic-like manifestations in the rodent model developed in the previous manuscript and assesses its response to olanzapine and

the combination of olanzapine and fluoxetine. This manuscript (**Manuscript B**) has been prepared for submission to *Frontiers in Psychiatry, Animal Models of Psychiatry* section (Frontiers Media SA).

**Chapter 5: Conclusion and Recommendations**

This chapter gives an overview and discussion of both manuscripts (Chapters 3 and 4) and also incorporates the findings presented in the addenda. A final conclusion and a report on the limitations as well as recommendations for future directions for this research close this chapter.

**Addenda (Additional data)**

Important data not included in the manuscripts are presented here.

**Addendum A** reports data on the behaviour of the model in the novel object recognition test (nORT) as well as plasma brain-derived neurotrophic factor. Response to treatments are also included in this section. This Addendum is presented as a brief research report.

**Addendum B** reports data on the model's response to olanzapine/fluoxetine and fluoxetine treatments in the forced swim test (FST) and prepulse inhibition (PPI) test, respectively.

**Addendum C** reports details of the methods used for the nORT, PPI behavioural tests, as well as the HPLC and ELISA analytical methods relating to **Manuscripts A** and **B**, as well as **Addendum A** and **B**.

References for Chapters 3 and 4 appear at the end of the respective chapters, while a bibliography for Chapters 1, 2, and 5 as well as Addenda A-C is listed at the end of the thesis under the heading "Bibliography".

**Appendix** (Supplementary documents relating to this study):

**Appendix A:** Co-author approval letters

**Appendix B:** Confirmation of submission of Manuscript A to *Journal of Affective Disorders*

**Appendix C:** Letter of ethics approval

**Appendix D:** Monitoring sheets used in this study

**Appendix E:** Congress proceedings

## 2. Problem Statement

There is a dearth of knowledge on the biology and pharmacology of psychotic depression. Research has given insight into its epidemiology (Crebbin *et al.*, 2008), pathophysiological hypotheses (Schatzberg *et al.*, 1985), cerebral aberrations (Vassilopoulou *et al.*, 2013), and has illustrated differences and similarities between MDpsy and other mood (bipolar disorder) and psychiatric (schizophrenia) disorders (Hill *et al.*, 2009). It has also illuminated the shortcomings of MDpsy diagnosis (Østergaard *et al.*, 2015) and its treatment (Wijkstra *et al.*, 2015).

The diagnosis of MD and MDpsy and the efficacy of treatment are quite subjective, being based largely on how a patient feels and scores on psychometric assessments. Indeed, the relevance of some of these assessments for MDpsy evaluation is debated (Ostergaard *et al.*, 2014). Despite "the usual suspects" like biogenic amines (serotonin (5-HT), dopamine (DA) and norepinephrine (NE)) and cortisol, the biological underpinnings of MDpsy remain uncertain (Croarkin, 2018), which underlies the lack of new drug development strategies for treating the condition. Another shortcoming is the lack of a valid animal model of MDpsy with which to identify novel drug targets and to test novel treatments. Thus, this study seeks to develop and validate a new animal model with robust translational validity for MDpsy, looking closely at how putative biological and behavioural markers of MDpsy correlate with response to typical drug treatment regimes.

The treatment of MD has traditionally been aimed at regulating monoaminergic systems. However, over time these antidepressants have proven to be less than ideal due to slow response times, numerous unfavourable side effects, the possibility of relapse, and inefficacy (Nestler *et al.*, 2002; Rosenzweig-Lipson *et al.*, 2007). Indeed, as many as 50% of patients fail to respond to a first-line antidepressant, which essentially renders 50% of the depressed population treatment-resistant. However, when the diagnosis of MD is further complicated by the presence of psychotic features, the outlook is even bleaker. Here as few as 17% of MDpsy patients treated with appropriate treatment reach and maintain remission for a year, with relapse rates ranging between 50% and

92% (Rothschild, 1996). Current treatments of MDpsy are based on physicians' extrapolations of drugs developed for depression and psychosis, while no drug treatments have been specifically developed for MDpsy (Rothschild, 2009). This maintains the lack of international consensus on the treatment of MDpsy but also underscores the need for new and more effective drugs or drug augmentation strategies that impact on appropriate biological pathways.

Treatment of MDpsy is more effective when using antidepressant/antipsychotic co-therapy, more specifically a SSRI plus an atypical antipsychotic (Nestler *et al.*, 2002; Vega *et al.*, 2000). In this regard, the use of fluoxetine/olanzapine co-therapy has been demonstrated to be effective in treating MDpsy (Shelton *et al.*, 2001), implicating the need to modulate both the serotonergic and dopaminergic systems; a given considering the pathophysiology of the disorder. However, the relationship between MDpsy and bipolar disorder (BD), MDpsy and schizophrenia, as well as schizophrenia and BD, has been confirmed on the basis of family history, possibility of a diagnostic conversion from MDpsy to BD, and overlaps in genetic markers (Domschke, 2013; Keller *et al.*, 2007; Ostergaard *et al.*, 2014).

The lack of a valid translational animal model has hindered progress into research relating to the drug treatment and diagnostic markers of MDpsy. Over the years, drug combinations that have demonstrated some efficacy in treating patients with MDpsy (Rothschild, 2013) have been tested in healthy rats (Horowitz *et al.*, 2003). However, an ideal animal model should resemble the disorder it represents in symptomology, biology and response to pharmacotherapy to better inform of effective therapies and potential diagnostic markers. Although models of TRD have recently been developed, e.g. Brand and Harvey (2017), Pereira *et al.* (2019), and Samuels *et al.* (2011), these models did not address the issue of psychosis occurring in depression. The closest animal model to MDpsy, at least in the co-occurrence of depression (manifested as hypo-activity) and mania (demonstrated by hyperactivity), is the ouabain model for BD (El-Mallakh *et al.*, 1995). However, it lacks other symptoms occurring in MDpsy. As of 2017, a simple PubMed search using the keywords: "animal model" AND "psychotic depression" yielded no published research describing the modelling of MDpsy in animals.

Considering the available evidence that MDpsy underscores the involvement of early life adversity as well as a genetic contribution that determines risk vs. resilience, this study aims to develop a

validated gene-x-environmental model of major depression with psychotic features by combining a genetic rat model of MD, namely, the Flinders Sensitive Line (FSL) rat (Overstreet, 1993), with a neurodevelopmental model of psychosis (schizophrenia) i.e. the social isolation rearing (SIR) model (Fone & Porkess, 2008). Indeed, we have earlier shown that SIR is able to induce cognitive and psychotic- (Moller *et al.*, 2013; Uys *et al.*, 2017), depressive- (Fone & Porkess, 2008) and anxiety-like (Regenass *et al.*, 2018) manifestations. With each model validated (face, construct, and predictive) for the specific disorder it intends to model, we hypothesise that exposing an animal that is genetically vulnerable to develop depressive-like symptoms (FSL rat) to a chronic early life neurodevelopmental stressor (post-weaning SIR) will result in depressive *and* psychotic-like behaviour, as well as reproduce known neuropathological biomarker changes akin to MDpsy, namely, elevated monoamines, hypercortisolaemia, and reduced basal plasma DBH. Moreover, we propose that these animals will exhibit resistance to standard antidepressant treatment, i.e. fluoxetine (FLX), slightly improved response to olanzapine (OLZ) alone, but marked response to a combination of olanzapine plus fluoxetine (OFC). Such an animal model of MDpsy would open the doors to investigating new treatment strategies as well as identifying novel biological and genetic markers.

## 3. Study hypotheses, questions, and aims

### 3.1 Hypothesis

The main hypothesis of this study is that combining a genetic model of depression, the FSL rat, with the social isolation rearing (SIR) model of schizophrenia, will result in an animal that exhibits bio-behavioural aberrations related to both depression and psychosis as well as display non-response or partial response to either fluoxetine or olanzapine used as monotherapies, but show improvement following a olanzapine/fluoxetine combination.

#### 3.1.1 Depressive-like behaviour of the model:

Specifically, the SIR-exposed FSL rat will exhibit depressive-like manifestations, particularly:

- Increased immobility in the forced swim test (FST)
- Reduced cognitive function (memory deficits) in the nORT

- Increased social withdrawal and social anxiety-like behaviour in the social interaction test (SIT)
- Reduced plasma dopamine-β-hydroxylase (DBH, DβH), and brain-derived neurotrophic factor (BDNF)
- Raised plasma corticosterone (CORT), interleukin-6 (IL-6), and tumour necrosis factor alpha (TNF-α)
- Reduced central NE, 5-HT, DA

### 3.1.2 Psychotic-like behaviour of the model:

Specifically, the SIR-exposed FSL rat will exhibit psychotic-like manifestations, particularly:

- Increase in locomotor activity in the open field test (OFT)
- Reduced cognitive function (memory deficits) in the nORT
- Suppression of sensorimotor gating function in the PPI test
- Raised plasma CORT, IL-6, and TNF-α
- Raised central 5-HT
- Disordered central NE and DA

### 3.1.3 SIR-exposed FSL rats will show strong similarities to clinical MDpsy:

- Aforementioned aberrations
- Raised central NE, 5-HT, and DA
- Treatment resistance, particularly:
  - Fluoxetine (FLX) will not improve depressive-like behaviour as explained in 3.1.1
  - Olanzapine (OLZ) will not improve psychotic-like behaviour as explained in 3.1.2
- Response (improvement in manifestations) to olanzapine/fluoxetine combination

## 3.2 Study questions

This project was designed to address the following study questions as they relate to the validation and translational application of an animal model of MDpsy:

Question 1:

1.1 Manuscript A: Will FSL rats demonstrate depressive-like behaviour and altered monoamine profile and plasma biochemistry compared to SD rats?

1.2 Manuscript A: Will the combination of a genetic animal model of depression (FSL rat) with a social isolation rearing (SIR) paradigm for schizophrenia result in a model resembling the behavioural and neurobiological changes observed in clinical TRD compared to SD and FSL animals?

1.3 Manuscript A: Will the resultant animal model (FSL-SIR) demonstrate non-response to chronic treatment with a standard of care drug i.e: fluoxetine, as observed in clinical TRD?

Question 2:

2.1 Manuscript B: Will SD-SIR rats demonstrate psychotic-like behaviour and altered monoamine profile and plasma biochemistry relative to SD rats?

2.2 Manuscript B: Will the resultant animal model (FSL-SIR) demonstrate behavioural and biochemical changes similar to those observed in clinical MDpsy?

2.3 Manuscript B: Will the resultant animal model (FSL-SIR) demonstrate poor response to chronic treatment with an antipsychotic drug indicated for schizophrenia i.e. olanzapine, as observed in clinical MDpsy?

2.4 Manuscript B: Will augmentation therapy, that is, the combination of fluoxetine and olanzapine result in improved behavioural and biochemical responses compared to either drug used alone?

### 3.3 Project aims

To address the study questions of the research project, the following aims are set in place:

1. Confirm the FSL rat as a model of depression relative to SD controls

a. Measure performance of FSL rats in the FST
b. Measure cortico-hippocampal monoamines: norepinephrine (NE), (5-HT), and DA (dopamine)
c. Measure plasma DBH, CORT, IL-6, and TNF-α
d. Confirm response to FLX treatment

2. Confirm SIR of Sprague-Dawley (SD) rats as a neurodevelopmental model of schizophrenia (SCZ)/psychosis relative to SD controls
    a. Measure sensorimotor gating performance of SIR-exposed (SD-SIR) rats in the PPI test
    b. Measure cortico-hippocampal monoamines: norepinephrine (NE), (5-HT), and DA (dopamine)
    c. Measure plasma DBH, CORT, IL-6, and TNF-α
    d. Confirm response to OLZ treatment

3. Establish validity of the gene-x-environment animal model of MDpsy (FSL-SIR) relative to saline-treated SD, FSL, and SD-SIR rats:
    a. measuring performance of FSL-SIR rats in the OFT to determine psychomotor agitation or retardation, FST to assess depressive-like behaviours, and to assess psychotic-like behaviours (sensorimotor gating deficits) in the PPI test
    b. evaluating performance of individual rats in the nORT to assess cognitive function
    c. assessing performance of rat pairs in the SIT to establish social withdrawal, social anxiety-like behaviour, and aggression
    d. determining whether the cortico-hippocampal concentrations of NE, 5-HT, DA and plasma DBH, CORT, IL-6, and TNF-α are similar to those observed in TRD/MDpsy
    e. ascertain whether or not the behavioural and neurobiological deviations seen in the FSL-SIR demonstrate resistance to 14-day FLX administered at a daily dose of 10 mg/kg
    f. establish whether or not the behavioural and neurobiological deviations seen in the FSL-SIR demonstrate resistance to 14-day OLZ, an atypical antipsychotic treatment, administered at a daily dose of 5 mg/kg
    g. determine whether or not FSL-SIR rats will exhibit improved response (behavioural and neurochemical) to co-administration of FLX (10 mg/kg) and OLZ (5 mg/kg) compared to either drug administered as monotherapy as observed in clinical MDpsy

h. compare bio-behavioural manifestations occurring in FSL-SIR rats to FSL and SD-SIR rats before and after treatment

## 4. Study design

Male SD and FSL rats were used in this study. These were weaned at 21 days of age (PND21) and randomly assigned to either social rearing or isolation rearing for 8 weeks. Animals received treatment for 14 days starting from PND63: socially-reared SD rats received saline; socially-reared FSL rats received saline or fluoxetine; socially-isolated SD rats were treated with saline or olanzapine; and socially-isolated FSL rats received saline, FLX, OLZ, or olanzapine+fluoxetine (OFC). Behavioural testing was conducted from PND72 and brain and blood tissue were collected at PND77 (Figure 1-1).

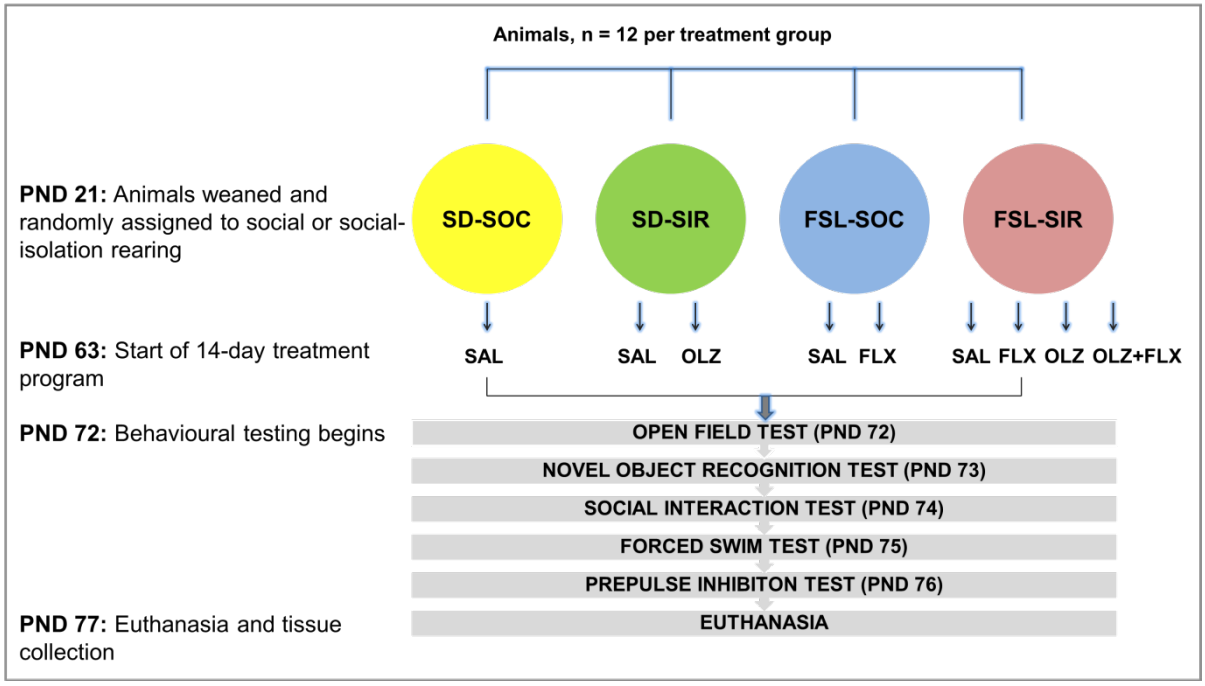


**Figure 1-1:** Graphical summary of the study layout.

The separate study design for Manuscripts A and B are now described.

# Chapter 1: Introduction

Section 1: Manuscript A

*Title:* **Flinders Sensitive Line rats exposed to post weaning isolation rearing are an animal model of treatment resistant depression**

For this section of the study, experimental groups were structured as below. Saline-treated SD rats served as a control to 1) saline-treated FSL rats to confirm the depressive-like profile of the FSL rat, and 2) saline-treated FSL-SIR rats to demonstrate treatment resistance. Saline-treated FSL rats served as controls to FSL-SIR rats to demonstrate more severe depressive- and social anxiety-like symptoms that would show a response to treatment different from FSL rats. Central and peripheral biomarkers were carefully selected based on their involvement in MD and TRD. The frontal cortex and hippocampus are implicated in MD. The prefrontal cortex is implicated in cognitive function (Ott & Nieder, 2019) as well as regulation of mood through its connection to the limbic region (Pandya *et al.*, 2012), while the hippocampus plays a role in emotion, neuroendocrine stress hormone regulation, and declarative memory (Nakahara *et al.*, 2018). Reduced cortico-hippocampal monoamines (NE, 5-HT, DA) coincide with the biogenic amine theory of depression. Deficits in NE are associated with social withdrawal; 5-HT deficits affect appetite and mood; and DA deficits are linked to impaired cognition and concentration (Jesulola *et al.*, 2018; Swanepoel *et al.*, 2018). Corticosterone, DBH, IL-6, TNF- α, and BDNF were measured in plasma to correlate them to clinical findings which are mainly based on fluid sample readouts. Elevations in CORT (cortisol/corticosterone), TNF-α and IL-6 predict antidepressant resistance (Perlman *et al.*, 2019) while low BDNF levels are observed in MD (Brand *et al.*, 2015). BDNF is also particularly relevant for mood disorders associated with cognitive dysfunction (Berk *et al.*, 2011; Brand *et al.*, 2015). Reduced DBH levels are associated with resistance to SSRIs including FLX (Willner & Belzung, 2015). This is explained in detail in Manuscript A.

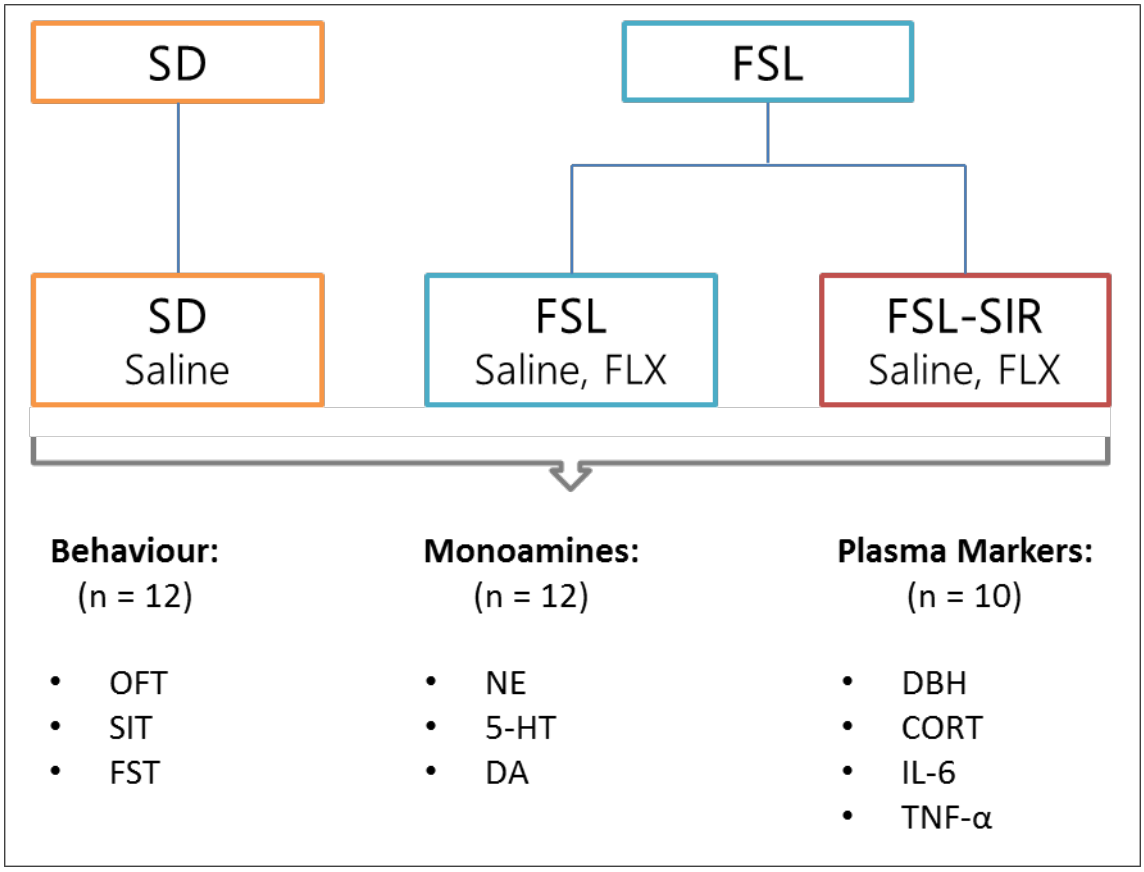


**Figure 1-2:** Manuscript A study layout. Post-weaned FSL rats were assigned to either social or isolation rearing. Rats received treatment from PND63. Behavioural assessments commenced from PND72 starting with the OFT. Monoamines were quantified from brains while plasma was used to quantify TRD-related biomarkers. Brains and blood were collected post-mortem (PND77).

Section 2: Manuscript B

*Title:* **Bio-behavioural validation of a novel neurodevelopmental animal model of treatment resistant depression: Response to olanzapine with/without fluoxetine**

For this section of the study, experimental groups were structured as below. Saline-treated SD rats served as a control to 1) saline-treated, socially-isolated SD rats to confirm the psychotic-like profile of SD-SIR rat, and 2) saline-treated FSL-SIR rats to demonstrate treatment resistance. Saline-treated SD-SIR rats served as controls to FSL-SIR rats to demonstrate more severe anxiety, psychotic- and social anxiety-like symptoms that would show, at best, partial response to OLZ compared to SD-SIR rats but enhanced response to OFC treatment compared to SAL treatment of

FSL-SIR. Central and peripheral biomarkers were carefully selected based on their involvement in psychosis (SCZ) and MDpsy. In this regard, the frontal cortex and hippocampus are both involved in SCZ and MD with frontocortical dysfunction underlying many of the cognitive and negative or affective symptoms of SCZ, while deficits in cognition are associated with hippocampal dysfunction (Ott & Nieder, 2019; Uys *et al.*, 2017). Disordered monoaminergic profiles translate to behavioural anomalies observed in MDpsy; in particular: reduced frontocortical DA is associated with psychosis; reduced cortical NE and 5-HT are associated with negative symptoms (e.g. social withdrawal), while raised NE and 5–HT are associated with positive symptoms (e.g. hallucinations) (Brisch *et al.*, 2014; Khalesi *et al.*, 2019; Meltzer, 1995; Weinstein *et al.*, 2017). Hypercortisolaemia and reduced DBH levels are associated with MDpsy (Schatzberg *et al.*, 1985). Elevations in the pro-inflammatory cytokines IL -6 and TNF–α are associated with SCZ and psychotic symptoms (Moller *et al.*, 2013). BDNF plays a role in cognitive function and reduced BDNF levels are associated with psychotic symptoms (Berk *et al.*, 2011; Brand *et al.*, 2015). CORT, DBH, IL-6, TNF- α, and BDNF were measured in plasma to correlate them to clinical findings which are mainly based on fluid sample readouts. This is explained in detail in Manuscript B.

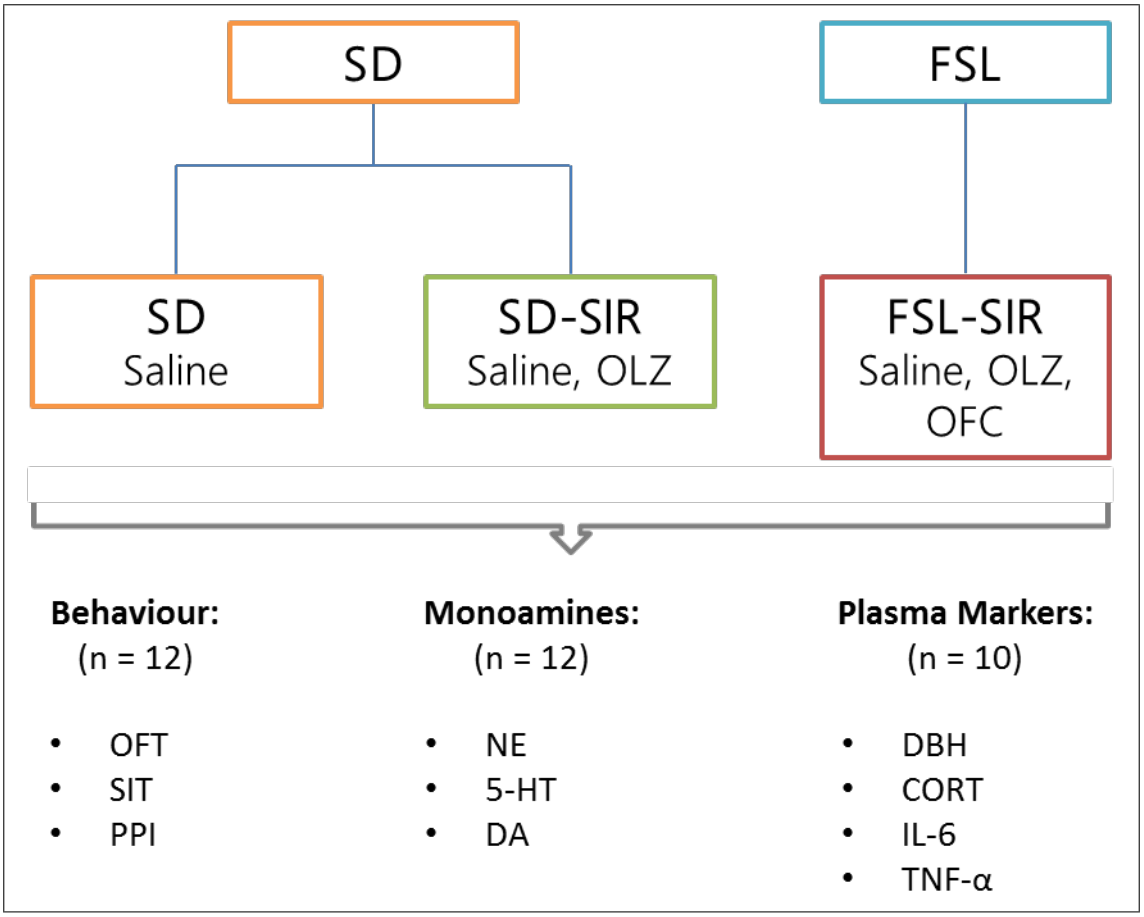


**Figure 1-3:** Manuscript B study layout. Post-weaned SD rats were assigned to either social or isolation rearing while FSL rats were reared in isolation from weaning. Rats received treatment from PND63. Behavioural assessments commenced from PND72 starting with the OFT. Monoamines were quantified from brains while plasma was used to quantify MDpsy-related biomarkers. Brains and blood were collected post-mortem (PND77).

## 5. Ethical considerations

This study was approved by the AnimCare animal research committee (NHREC reg. no. AREC-130913-015) of the North West University (NWU) (Ethics approval number: NWU-00150-18-S5). Animals were bred, supplied, and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform of the NWU.

The research was executed in adherence to the 3R guidelines: Replace, Refine, and Reduce (Fenwick *et al.*, 2009).

Replace: Since the aim of the study was to develop and validate an animal model of MDpsy, animals (rodents) were an obligatory requirement of the study.

Reduce: A power analysis for sample size determination was implemented to determine an optimal number of animals per study group. This ensured that the least number of animals would be used in the study while ensuring adequate power to detect statistical significance. Furthermore, each group of animals was used for both behavioural and neurochemical analyses. This further served to reduce the number of animals for the study.

Refinement: To develop and validate an animal model that exhibited symptoms of both depression and psychosis, two separate translational animal models were combined. The Flinders Sensitive Line (FSL) rats are an established genetic model of depression (Overstreet & Wegener, 2013), while social isolation rearing is an established neurodevelopmental model of schizophrenia/psychosis (Moller *et al.*, 2015). Also, with the aim of keeping animal numbers low, only male rats were used in the study. While females are more prone than males to develop depression (Slattery & Cryan, 2014), no gender differences were observed in the incidence and prevalence of psychotic depression (Bogren *et al.*, 2018). The exclusion of female rats from the study was further supported by the apparent differences in HPA-axis activity as well as antidepressant metabolism and subsequent response observed between female and male rats (Goel *et al.*, 2014; Kokras & Dalla, 2017).

In the interest of demonstrating face validity of this model, several bio-behavioural tests were conducted. The tests were arranged from least to most stressful (Mokoena *et al.*, 2015) and conducted on different days to allow the animals rest so as to prevent excessive stress on the animals.

The route of administration of the drugs was also carefully considered since the experience of an injection is considerably stressful to the animals. The least stressful but most reliable route of administration of treatment for this research was via subcutaneous injection.

## 6. Expected results

The expected results of the current study are presented and discussed in Table 1 in response to the main questions as presented in the *Study questions* section. Table 1 reiterates the study questions and indicates the section of the thesis in which the question is investigated.

**Table 1:** The study questions explored in the thesis with their expected results.

| Study question | Expected result |
|---|---|
| **(1.1)**<br>Will FSL rats demonstrate depressive-like behaviour and altered monoamine profile and plasma biochemistry compared to SD rats?<br>**Manuscript A** | FSL rats will demonstrate depressive-like and social anxiety-like behaviours compared to SD controls. FSL rats will present with altered regional brain monoamine profile and plasma biochemistry compared to SD rats. These altered behaviours and biochemistry will be normalised by FLX treatment. |
| **(1.2)**<br>Will exposing the FSL rat SIR result in a model resembling the bio-behavioural changes observed in clinical TRD compared to SD and FSL animals?<br>**Manuscript A** | FSL plus SIR will result in enhanced symptoms of depression and social anxiety-like behaviour. FSL-SIR rats will demonstrate regional brain monoamine alterations and changes in TRD-related biomarkers compared to SD and FSL rats. |
| **(1.3)**<br>Will the resultant animal model (FSL-SIR) demonstrate non-response to chronic treatment with FLX, as observed in clinical TRD?<br>**Manuscript A** | FSL-SIR rats will be non-responsive or, at best, partially responsive to FLX. |

| | |
|---|---|
| **(2.1)**<br>Will SD-SIR rats demonstrate psychotic-like behaviour and altered monoamine profile and plasma biochemistry relative to SD rats?<br>**Manuscript B** | SD-SIR rats will demonstrate PPI and social impairments compared to SD controls. SD-SIR rats will exhibit alterations in regional brain monoamines and psychosis-related biomarkers compared to SD control animals. These deviations will be normalised by OLZ treatment. |
| **(2.2)**<br>Will the FSL-SIR rat exhibit behavioural and biochemical changes similar to those observed in clinical MDpsy?<br>**Manuscript B** | FSL-SIR rats will exhibit psychosis- and enhanced anxiety-like behaviours, as well as social anxiety. FSL-SIR rats will demonstrate alterations in regional brain monoamines and MDpsy-related biomarkers compared to SD and SD-SIR rats. |
| **(2.3)**<br>Will the FSL-SIR rat exhibit bio-behavioural response to chronic treatment with OLZ, as observed in clinical MDpsy?<br>**Manuscript B** | FSL-SIR rats will, at best, exhibit partial response in the above-mentioned bio-behavioural parameters to an atypical antipsychotic, OLZ. |
| **(2.4)**<br>Will combination therapy, that is, FLX plus OLZ result in improved bio-behavioural responses compared to either drug used alone?<br>**Manuscript B** | The combination of FLX and OLZ will result in improvement in the above-mentioned bio-behavioural alterations in the FSL-SIR rat compared to either drug used alone. |
| **(1.2, 1.3, 2.2, 2.3)**<br>Will the FSL-SIR rat exhibit worse cognitive dysfunction vs. FSL and SD-SIR rats?<br><br>Will the FSL-SIR rat's cognitive performance be altered by FLX, OLZ, or OFC treatment?<br>**Addendum A** | FSL-SIR rats will show worse deficits in memory than FSL rats. FLX will improve memory in FSL rats but will have no effect in FSL-SIR rats. FSL-SIR rats will show equally poor memory performance as in SD-SIR rats. OLZ will improve cognitive dysfunction in the SD-SIR rat, but partially improve dysfunction in FSL-SIR rats. OFC will resolve cognitive dysfunction in the FSL-SIR rat. |

| | |
|---|---|
| **(1.2, 1.3, 2.2, 2.3)**<br>How will BDNF plasma levels in the FSL-SIR rat compare to those of FSL and SD-SIR rats?<br><br>Will BDNF plasma concentration in the FSL-SIR rat be altered by FLX, OLZ or OFC?<br><br>**Addendum A** | Plasma BDNF levels will be reduced in FSL, SD-SIR, and FSL-SIR rats. FLX and OLZ will raise BDNF levels in the FSL and SD-SIR rat, but will partially reverse this deficit in FSL-SIR rats. OFC will markedly improve memory deficits in FSL-SIR rats. |
| **(1.2, 1.3, 2.2, 2.3, 2.4)**<br>Will the FSL-SIR model exhibit depressive-like behaviour and will it be responsive to either OLZ or OFC treatment?<br><br>Will the FSL-SIR model exhibit psychotic-like behaviour and will it be responsive to FLX treatment?<br><br>**Addendum B** | Depressive-like behaviour in FSL-SIR rats will be partially-responsive to OLZ, but fully reversed by OFC. Psychotic-like behaviour in FSL-SIR rats will not be responsive to FLX treatment. |

# CHAPTER 2: LITERATURE REVIEW

## 1. Introduction

Depression as we know it was largely defined and described by the works of Cassidy *et al.* (1957) who set out the criteria for a diagnosis of depression. These criteria were later slightly modified by Feighner *et al.* (1972) and then the American Psychiatric Association (APA) to form the DSM-III diagnosis criteria of depression in 1980 (APA, 1980). The diagnostic criteria have remained intact with the DSM-V exclusion of bereavement from the diagnostic criteria being the only amendment made since (APA, 2013). Hamilton (1960) developed and refined a rating scale (Hamilton Rating Scale of Depression, HRSD) of 17 items to quantify the severity of depressive symptoms. The Montgomery–Åsberg Depression Rating Scale (MADRS) was developed as an adjunct to the HRSD and is more sensitive to antidepressant-induced symptom changes (Montgomery & Asberg, 1979). Currently, depression is described as a mood disorder characterised by the presence of a predefined set of diagnostic criteria, not including aetiology, occurring for a defined period of time (Paykel, 2008).

Despite the relative newness of this definition, depression, and the description of its symptoms have been rather consistent for over 2500 years (Horwitz *et al.*, 2016).

Hippocrates (422–377 BC) described depression as "melancholia" – a term which encompassed all pathological states of depression – and defined it as "fear [anxiety] or depression that is prolonged" and presented with symptoms of: "aversion to food, despondency, sleeplessness, irritability, restlessness" (Horwitz *et al.*, 2016; Jackson, 1978). Melancholia was associated with blackness of mood and suicidal impulses, and paranoid tendencies such as sullen suspiciousness (Horwitz *et al.*, 2016) and thus aptly characterised as "delirium without a fever" by Du Laurens (1938) as his predecessor, Galen (129–216 AD). Galen and Du Laurens (1938) noted that accompanying depressive symptoms, melancholic patients were plagued by delusions and irrational fears citing some patients' fear that Atlas would grow weary from carrying the world on his shoulders and consequently drop it or simply vanish, leading Du Laurens (1938) to conclude that those suffering from melancholia had "disturbed imagination".

# Chapter 2: Literature review

The ancient Greeks viewed mental illness in terms of the four basic humors: phlegm, blood, yellow bile and black bile (Horwitz *et al.*, 2016; Telles-Correia & Marques, 2015). Health reflected a balance between the four humors and thus disease was credited to an imbalance of any of the humors (Horwitz *et al.*, 2016). This is somewhat mirrored in light of aetiological theories of neurochemical imbalances in depression and psychosis. Hippocrates attributed melancholia to an excess of black bile and further suggested that this particular imbalance negatively influenced, not just the mind, but also the relationship between the sufferers and their environments (Horwitz *et al.*, 2016; Jackson, 1978; Telles-Correia & Marques, 2015). Aristotle (384-322 BC) made the important contribution of distinguishing normal sadness from pathological sadness (depression) stating that the black bile in the melancholic patient is "cold beyond due measure" and thus "produces groundless despondency" (Horwitz *et al.*, 2016). The implication being that sadness resulting from the ups and downs of life i.e. disappointment or death of loved ones, was excluded from this diagnosis. This further distinguished reactive (short-lived response to psychosocial issues) sadness from endogenous (severe, chronic, prolonged) depression (Horwitz *et al.*, 2016)

In both the DSM-I and DSM-II, the diagnosis of melancholia was well-aligned to that defined by Hippocrates making melancholic (psychotic or endogenous) depression central to psychiatric theories, research, and practice (Horwitz *et al.*, 2016). This changed with the introduction of the DSM-III and its drastic redefinition of depression and criteria for its diagnosis which essentially made Hippocrates' "black bile" melancholia a subtype of major depressive disorder (depression, MD) (Horwitz *et al.*, 2016; Paykel, 2008) and MD the more important diagnosis. This unified definition of depression paved the way for the expansive use of antidepressants (Grob, 2012; Horwitz *et al.*, 2016). However, a downfall of this unified definition was by largely ignoring the many years invested into the study of melancholia by the ancient physicians; it seemingly somewhat negated the extreme complexity of the disorder and prematurely narrowed the focus of the possible causes of depression and its treatment, putting researchers and patients in the unfortunate position of still not understanding the neurobiological and genetic foundations of depression despite years of study (Nestler *et al.*, 2002). Indeed, many clinicians and researchers are of the opinion that the current DSM-V diagnostic criteria, being atheoretical and not incorporating new research trends into the neurobiological underpinnings of the disorder, has not only hamstrung a better understanding of its complexity but has effectively restrained research into better treatments (Szechtman *et al.*, 2020).

The discovery of the tricyclic antidepressants (TCAs) and the monoamine oxidase inhibitors (MAOs) as antidepressants was instrumental in postulating the monoamine hypothesis of depression which gave valuable insight into the neurobiology of depression and a clear direction for the development of subsequent antidepressants, namely, the selective serotonin reuptake inhibitors (SSRIs) (Hillhouse & Porter, 2015; López-Muñoz & Alamo, 2009). However, despite the value to research these discoveries made, these antidepressants have remission rate of less than 60% (Hillhouse & Porter, 2015) in addition to their slow onset of action ranging from a few weeks to months (Fava, 2003). Moreover, advances since these initial drug discoveries in the 1950s – 1980s have been modest (Nestler *et al.*, 2002). Depression thus remains an incapacitating, and multifaceted mental illness, the treatment of which is complicated by the heterogeneity of the affected populations in terms of gene-environment interactions, symptoms and occurrence of co-morbidities (Hollis & Kabbaj, 2014; Keller *et al.*, 2007; Rosenzweig-Lipson *et al.*, 2007).

One such co-morbidity complicating diagnosis and treatment is that of psychosis (hallucinations and/or delusions). Psychotic features occurring in depression (Hippocratic melancholia) results in a form of depression more severe than MD with distinct disease progression and response to treatment, poor prognosis, exaggerated symptomology, morbidity, and mortality (Bijanki *et al.*, 2014; Busatto, 2013; Keller *et al.*, 2007).

## 2. Epidemiology

Depression is a major, modern-day healthcare challenge affecting, on average, between 4.4 and 20% of the general population (Liu *et al.*, 2012; WHO, 2017). In South Africa, the lifetime prevalence is recorded at 9.7% (Tomlinson *et al.*, 2009). This figure increases notably in in-patients (Wang *et al.*, 2017), in rural areas, and among those with low socioeconomic status (Tomlinson *et al.*, 2009). Depression is recurrent and every episode increases the risk for later occurrences (Liu & Alloy, 2010). It is associated with lower quality of life and compromised cognitive function (Rubin *et al.*, 2018; Wang *et al.*, 2017); neuronal and endocrine abnormalities (Krishnan & Nestler, 2011; Nestler *et al.*, 2002); as well as substantial morbidity and mortality with close to 800000 suicide deaths a year (Sullivan *et al.*, 2000; WHO, 2017). The World Health Organisation ranked it as the single largest contributor to global disease disability (WHO, 2017) and it has been predicted to become the second leading cause of death by the year 2020 (Hollis & Kabbaj, 2014; Palazidou, 2012;

Rosenzweig-Lipson *et al.*, 2007). When the complication of co-occurring psychosis is added to the diagnosis, the epidemiological picture becomes bleaker.

Ohayon and Schatzberg (2002) conducted an epidemiological study in a European population aged 15-100 years and found that of the participants who fulfilled DSM-IV criteria for major depression, almost 19% suffered psychotic features resulting in a MDpsy prevalence of 0.4% (Ohayon & Schatzberg, 2002). Johnson *et al.* (1991) estimated that 14.7% of US patients with major depression reported a history of psychotic symptoms, translating to a lifetime prevalence of 0.6% (Johnson *et al.*, 1991). In the context of hospital in-patients admitted for depression, 25% have been found to suffer from MDpsy, the frequency of which drastically increases as a function of age, varying between 24% and 53% in populations over 60 years (Rothschild, 2013).

Yet, despite the relative frequency of MDpsy, it remains underdiagnosed and inadequately treated (Crebbin *et al.*, 2008; Rothschild, 2013), while its underlying neurobiology is grossly under-investigated. In both emergency cases and in in-patient settings, the diagnosis of MDpsy is often missed (Rothschild, 2009), as exemplified in a study by Østergaard *et al.* (2015) which documented that 27% of hospitalised patients with MDpsy were incorrectly diagnosed. In fact, the psychotic component of the illness is often completely missed (Rothschild *et al.*, 2008).

### 3. Symptomology and Diagnosis

MDpsy is a diagnostically stable, albeit more severe subtype of major depression that is associated with a high risk of relapse (Bijanki *et al.*, 2014; Crebbin *et al.*, 2008; Gournellis *et al.*, 2011; Rothschild, 1996).

A MDpsy diagnosis is based on the presence of two or more major depressive episodes (MDE) separated by an interval of at least two months (during which the criteria for MD are not met) *and* the occurrence of delusions and/or hallucinations that are either mood-congruent (related to guilt or sadness) or mood-incongruent (persecutory or paranoid) during MDEs (APA, 2013; Tonna *et al.*, 2012).

According to the DSM-V, a diagnosis of MD requires the individual to present with depressed mood and/or loss of interest or pleasure (APA, 2013) for a period exceeding two weeks as well as at least five of the symptoms listed in Table 2-1.

**Table 2-1:** Diagnostic criteria for major depression (APA, 2013)

| |
|---|
| Severe depressed mood |
| Anhedonia (including social withdrawal) |
| Psychomotor agitation/retardation |
| Feelings of worthlessness or inappropriate guilt |
| Weight changes |
| Diminished ability to think or concentrate, or indecisiveness |
| Insomnia or hypersomnia |
| Fatigue or lethargy |
| Recurrent thoughts of death or suicide (including attempts) |

MDpsy patients typically suffer more intense depression; cognitive disturbance deficits related to executive function, verbal declarative memory, and attentional performance; psychomotor agitation or retardation and more intense feelings of guilt than MD patients (Keller *et al.*, 2007; Nelson *et al.*, 1998; Schatzberg, 2003). Furthermore, MDpsy patients experience delusions that are characteristically paranoid or somatic (hypochondria) in nature, and/or somatic, auditory, or visual hallucinations (Gaudiano *et al.*, 2009; Keller *et al.*, 2007; Schatzberg, 2003). Other features of MDpsy include little to no diurnal variation of depressive symptoms, increased suicidal ideation, constipation, and a family history of especially bipolar disorder (BD) (Gaudiano *et al.*, 2009; Keller *et al.*, 2007; Ostergaard *et al.*, 2014a; Rothschild, 2013; Schatzberg, 2003). Higher cortisol levels and greater HPA axis activity are also observed in MDpsy compared to MD (Bijanki *et al.*, 2014; Keller *et al.*, 2007).

## 4. Aetiology and Pathophysiology

Major depression (MD) is directly related to the human emotions of sadness and mourning; however, in the absence or the end of the cause of these emotions, or in cases where they are disproportionate to their nature, treatment is required (Villas Boas *et al.*, 2019). The symptoms of MD are consequences of inherent (genetic) and environmental factors (life experiences and lifestyle) that disrupt the proper regulation of several neurotransmitters and metabolic systems (Hamon & Blier, 2013; Villas Boas *et al.*, 2019).

The exact aetiology and pathophysiology of MD remains elusive, although MD is associated with numerous biological abnormalities such as dysregulated hypothalamic-pituitary-adrenal (HPA) axis activity (Lamers *et al.*, 2013), circadian dysrhythmias (Villas Boas *et al.*, 2019), metabolic abnormalities (Renshaw *et al.*, 2009), as well as aberrant signalling of neurotrophic (Krishnan & Nestler, 2011), monoaminergic (Brand *et al.*, 2015), and inflammatory agents (Steptoe *et al.*, 2007).

While MD is often treatable, over 50% of patients do not respond to antidepressant therapy and so MD treatment is not totally curative (Hamon & Blier, 2013). Although prior and unresolved psychosocial stress, including both gene and environmental factors, are recognised contributing factors, the deeper underlying mechanisms as to why some people develop MD and others not (Seki *et al.*, 2018) remains unknown. Furthermore, it is not known why some of MD sufferers are treatment-resistant; however, some risk factors have been identified. They include: 1) late age of onset of depression and family history (Kornstein & Schneider, 2001); 2) non-response to first antidepressant therapy (Rosenzweig-Lipson *et al.*, 2007); 3) history of abuse (sexual, physical, neglect) (Nasca *et al.*, 2018); 4) personality traits (Takahashi *et al.*, 2013) and personality disorder (Souery *et al.*, 2007); 5) comorbid psychiatric disorders (especially anxiety), insomnia, pain sensitivity, and gender (Cepeda *et al.*, 2018); 6) current risk of suicide (Rosenzweig-Lipson *et al.*, 2007); high recurrence rates (Rizvi *et al.*, 2014); and 8) undetected symptoms including delusions (in psychotic depression) (Schatzberg, 2003).

Psychosis renders MD a form of treatment resistant depression (TRD) due to improper treatment (resulting from misdiagnosis, incorrect dosing, or too short duration of treatment) but also because of the neurochemical and structural similarities that exist between TRD and psychotic depression (MDpsy).

### 4.1 Structural and functional abnormalities

Structural and functional neuroimaging have shown that neurological differences exist between healthy and depressed brains (Dunlop & Mayberg, 2017). Furthermore, structural differences are evident between the depressed and the psychotically depressed (and TRD) brain (Bijanki *et al.*, 2014; Simpson *et al.*, 1999; Skaf *et al.*, 2002).

The link between hippocampal atrophy and major depression is well-established (Bijanki *et al.*, 2014; Lange & Irle, 2004; Leuchter *et al.*, 2010; MacQueen & Frodl, 2011; Skaf *et al.*, 2002), with Bijanki *et al.* (2014) further demonstrating small hippocampal volume to be 10.1% more common in psychotically depressed patients compared to healthy controls (Bijanki *et al.*, 2014). Hippocampal volume has recently been observed as a differentiating factor between treatment responders and non-responders, with a smaller baseline hippocampal volume predicting poor clinical outcome (Malykhin *et al.*, 2010) whereas larger hippocampal volumes predict positive response to pharmacotherapy (Breitenstein *et al.*, 2014). Maller *et al.* (2012) further reported a unique finding of specifically reduced volume in the tail regions of the hippocampus in patients with either TRD or schizophrenia (Maller *et al.*, 2012). Skaf *et al.* (2002) determined that a small insular cortex volume discriminated psychotic from non-psychotically depressed patients.

Frodl *et al.* (2008) conducted a prospective, longitudinal study in which an association was found between larger hippocampal volumes and better response to antidepressant therapies as well as to lower relapse rate over 3 years (Frodl *et al.*, 2008). Aside from brain region volume serving as a predictor for treatment responsiveness, glucose metabolism in the brain also serves as an additional discriminator of responders from non-responders, specifically: the association between hypermetabolism and non-response to antidepressant treatment (Mayberg *et al.*, 2000).

Skaf *et al.* (2002) imaged the brains of two groups of depressed patients: 1) severe depression with psychotic features; and 2) severe depression without psychotic features using single photon emission computed tomography (SPECT). This study revealed significant decreases in regional cerebral blood flow (rCBF) in the left subgenual cingulate cortex (sgACC) relative to both non-psychotic patients and healthy controls. Additionally, reduced glucose metabolism has been implicated in a localised reduction in synaptic activity or by a reduction in grey matter volume (Drevets *et al.*, 1997).

Smaller rACC volume is associated with elevated depressive symptoms and is predictive of poor treatment response and more frequent hospitalisation (Bijanki *et al.*, 2014; Leuchter *et al.*, 2010; Pizzagalli, 2011; Webb *et al.*, 2018) while hypometabolism (compared to controls) in this region was not altered by antidepressant treatment in non-responders (Brannan *et al.*, 2000; Mayberg *et al.*, 2000). Inactivation of the rACC by deep brain stimulation also elicits quick and long-lasting antidepressant effects (Belzung *et al.*, 2015). Furthermore, decreased activity in the left ventro-lateral prefrontal and anterior cingulate cortex is shown to be related to increased negative self-image (Sperduti *et al.*, 2013).

### 4.2 State of Inflammation

The cytokine hypothesis proposed by Smith (1991) as "the macrophage theory of depression" was one of the first steps in highlighting a bidirectional link between depression pathogenesis and immune dysfunction.

Psychological stress has been shown to increase the production of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) which are associated with the development of depressive symptoms (Bluthe´ *et al.*, 1992; Seki *et al.*, 2018). Chronically elevated levels of these cytokines in peripheral blood are regarded as reliable biomarkers of depression where elevated baseline levels of these pro-inflammatory cytokines could potentially predict non-response to antidepressant treatment (Brand *et al.*, 2015; Eller *et al.*, 2008; Perlman *et al.*, 2019; Seki *et al.*, 2018; Sukoff Rizzo *et al.*, 2012; Villas Boas *et al.*, 2019; Yoshimura *et al.*, 2009).

IL-6 levels are negatively correlated to grey matter volume of the hippocampus in healthy adults suggesting an influence of inflammation on brain structure and function (Marsland *et al.*, 2008; Mondelli *et al.*, 2011). This relationship was mirrored by a preclinical study in mice that revealed that overexpression of IL-6 in the frontal cortex and hippocampus following intracranial administration blunted antidepressant response to fluoxetine (Sukoff Rizzo *et al.*, 2012). Moreover, IL-6 levels are positively correlated to the severity of depression (Hodes *et al.*, 2016; Yoshimura *et al.*, 2009).

Elevated serum levels of TNF-α have been observed in TRD; however, whether or not these levels are affected by antidepressant therapy is contested. One clinical study showed TNF-α levels were not lowered following a 12-week course of escitalopram (Eller *et al.*, 2008) and was supported by a

meta-analysis by Hannestad *et al.* (2011). In contrast, the meta-analysis by Strawbridge *et al.* (2015) showed a decrease in TNF-α in treatment-responsive patients following antidepressant treatment. This is further supported by studies that have shown an improvement in depressive symptoms in TRD (Raison *et al.*, 2013) and MD (Tyring *et al.*, 2006) patients following anti-inflammatory administration to treat disorders of immune dysfunction or as adjunct therapy to antidepressant treatment (Perlman *et al.*, 2019; Seki *et al.*, 2018).

Inflammation is closely linked to oxidative stress and has been implicated in the pathophysiology of schizophrenia (a disorder marked by psychosis) (Brand *et al.*, 2015). The activated immune system, specifically interferon-γ (IFN-γ), TNF-α, induce the enzyme indoleamine 2,3-dioxygenase (IDO) which increases kynurenic acid production which in turn affects glutamatergic and serotonergic neurotransmission (Brand *et al.*, 2015; Dantzer *et al.*, 2008; Müller *et al.*, 2015). IDO activation diverts tryptophan metabolism to quinolinic acid and away from 5-HT, thus paving the way for psychiatric manifestations driven by an increased glutamate:serotonin ratio (Moller *et al.*, 2015). Increased quinolinic acid is not only neurotoxic in its own right (Moller *et al.*, 2015), but can directly modify nicotinamide adenine dinucleotide ($NAD^+$) levels and thus adversely affect mitochondrial energy balance (Braidy *et al.*, 2011; Lugo-Huitrón *et al.*, 2013). This is described in Figure 1. Elevated cerebrospinal fluid (CSF) levels of kynurenic acid, an N-methyl-D-aspartate (NMDA) receptor antagonist, have been noted in both schizophrenic patients and in animal models of the disorder (Müller *et al.*, 2015). Elevated levels of pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-1, IL-6 and IL-10 are associated with schizophrenia; in particular, high CSF IL-1 levels are associated with acute psychosis while IL-6 is significantly raised in early and late stage schizophrenia (Brand *et al.*, 2015; Müller *et al.*, 2015).

Sustained, elevated levels of inflammation are a contributing factor in treatment resistance. Furthermore, Chang *et al.* (2012) reports that chronic low-grade inflammation could damage the dopaminergic neurons that correspond with cognitive functions such as psychomotor speed, memory, and executive cognitive function.

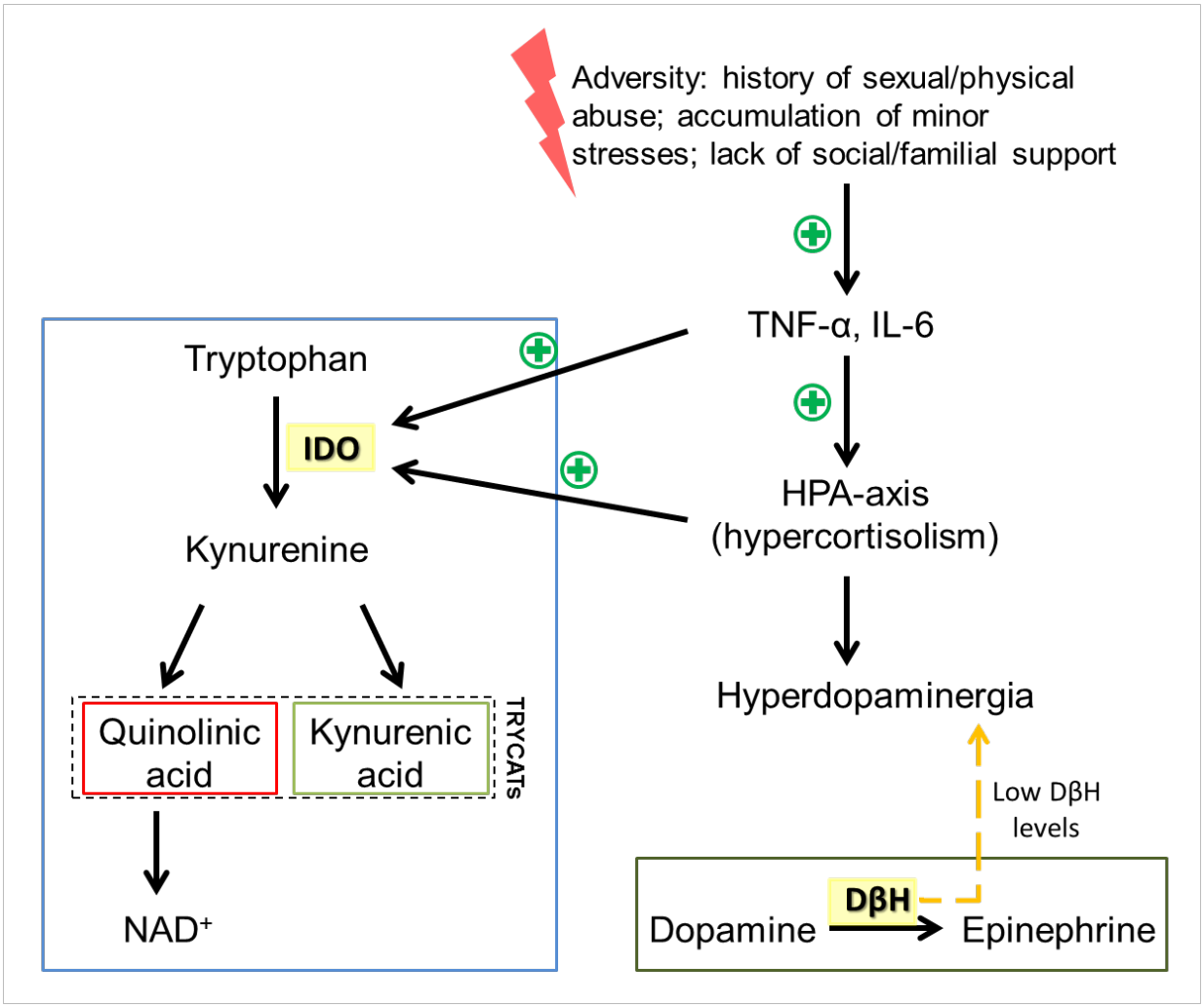


**Figure 2-1:** Simplified diagram of the kynurenine pathway and its relation to inflammation and the HPA-axis. Sustained stress increases pro-inflammatory cytokine production and excessively stimulates the HPA-axis resulting in increased dopamine metabolism and IDO activity producing kynurenic and quinolinic acid and modifying neurotoxic $NAD^+$ levels. Reduced DBH further contributes to hyperdopaminergia.

### 4.3 Hypercortisolaemia and Hyperdopaminergia

Mechanistically, chronic stress including negative life events (such as bereavement), a history of physical, emotional, and sexual abuse, unemployment, living with or raising multiple preschool children, as well as perceived lack of social or familial support, may activate the aforementioned inflammatory response (Perlman *et al.*, 2019; Seki *et al.*, 2018; Willner *et al.*, 2013). At high levels, the stress hormone, cortisol, is strongly associated with antidepressant resistance as well as with improved treatment response (Perlman *et al.*, 2019). A failure to suppress cortisol after dexamethasone suppression testing (DST), indicating a lack of response to negative feedback of the HPA-axis, could be a predictor of antidepressant non-response (Breitenstein *et al.*, 2014).

Dexamethasone suppression failure is associated with the development of psychotic symptoms (Rosenblat *et al.*, 2015), as determined by a meta-analysis of 14 studies that compared dexamethasone suppression in psychotic depression (MDpsy) vs. MD patients. The latter study showed high rates of non-suppression of cortisol following dexamethasone administration in 64% of MDpsy patients versus 41% of MD patients (Nelson & Davis, 1997) as well as exceptionally high cortisol levels after dexamethasone challenge (Carroll *et al.*, 1980; Mendlewicz *et al.*, 1982; Schatzberg *et al.*, 1985). Hypercortisolemia is strongly associated with poor clinical outcome, that is, poor response to tricyclic antidepressants (Thase, 2014), psychological treatment (Fischer *et al.*, 2017), and high relapse (Ribeiro *et al.*, 1993; Rothschild, 2013). Incidentally, schizophrenic patients do not exhibit similar rates of dexamethasone suppression failure (Schatzberg *et al.*, 1985).

Hypersecretion of cortisol as a consequence of psychological stress or a dysregulated HPA-axis stimulates dopamine metabolism in the striatal cells proportionally due to the interconnections between HPA-axis and the subcortical dopamine systems (Fleming *et al.*, 2004). This phenomenon is suggested to underlie both schizoaffective disorders and schizophrenia (Fleming *et al.*, 2004). Schatzberg *et al.* (1985) hypothesised that hypercortisolaemia results in hyperdopaminergia in MDpsy, a statement supported by both Posener *et al.* (1999) and Rothschild *et al.* (1984). The mechanism by which this occurs is unclear, although this relationship is not seen in healthy subjects (Wand *et al.*, 2007). Preclinical studies show that stress glucocorticoids increase mesolimbic DA and in particular that high cortisol secretors are also high DA secretors (Wand *et al.*, 2007). Furthermore, Pruessner *et al.* (2004) showed increased ventral striatal DA release in response to a psychosocial stessor in humans who reported poor early life maternal care. Hyperdopaminergia in MDpsy is the oldest neurobiological model of psychosis and plays a role in the experience of delusions (Schatzberg *et al.*, 1985; Tost *et al.*, 2010). It is based mainly on the observation that MDpsy patients were reported to respond poorly to tricyclic antidepressants until an antipsychotic was added to the treatment regimen (Kroessler, 1985; Schatzberg *et al.*, 1985). A dysfunction in the HPA-axis is also evident in the design of TRD models in animals, such as chronic adrenocorticotrophin (ACTH) adminstration in rats (Pereira *et al.*, 2019), while the strong association between a severe anxiety disorder, such as posttraumatic stress disorder (PTSD), and TRD has also been exploited as a theoretical basis from which to develop a translationally relevant TRD model in rodents (Brand & Harvey, 2017a, 2017b).

Elevated glucocorticoid levels are also linked to flattened diurnal rhythms which is suggested to impede the ability of SSRI's to raise 5-HT transmission mediated, in particular, by the serotonin-1A receptor (5-$HT_{1A}$) (Samuels *et al.*, 2011). Elevated cortisol levels during quiescent hours as a result of disordered HPA-axis function contribute to the defective action of the circadian timing system in MDpsy (Keller *et al.*, 2006). In MD, altered circadian rhythm is due to disordered functioning of the suprachiasmatic nucleus (Villas Boas *et al.*, 2019). Altered circadian rhythm indirectly disturbs functioning of mid-brain monoamine centres, in particular the ventral tegmentum (DA), raphae nucleus (5-HT) and locus coeruleus (NA) (Harvey & Slabbert, 2014). These changes eventually drive many of the biogenic amine-mediated bio-behavioural manifestations evident in mood and psychotic disorders (Harvey & Slabbert, 2014).

### 4.4 Reduced Dopamine-β-hydroxylase

Dopamine-β-hydroxylase (DBH) is an enzyme located in the secretory vesicles of neurons producing epinephrine and norepinephrine (NE) where it catalyses the conversion of DA to NE (Cubells *et al.*, 1998; Schatzberg *et al.*, 1985). MDpsy is strongly associated with significantly lowered CSF and serum levels of DBH (Domschke, 2013; Schatzberg *et al.*, 1985). In contrast, MD has been reported to have significantly higher levels of DBH (Cubells *et al.*, 2000; Schatzberg *et al.*, 1985) or similar levels compared to healthy controls (Keller *et al.*, 2007). These low levels could, in part, explain the elevated levels of CSF HVA (Nelson & Davis, 1997; Schatzberg *et al.*, 1985) which are reported to reflect psychomotor activity rather than delusion and mood (Duval *et al.*, 2006) in psychotically depressed patients. Moreover, reduced DBH levels have been confirmed by Cubells *et al.* (2000) to be a stable, genetically controlled trait. Schatzberg *et al.* (1985) argued that these levels do not normalise with improvement in health making it a potential trait biomarker for psychotic depression. Hamner and Gold (1998) explain that altered DBH may represent genetic vulnerability to developing psychiatric disorders such as MDpsy, MD, or psychotic bipolar depression. Furthermore, low DBH activity could be a consequence of neglect or abuse at critical periods of development (Hamner & Gold, 1998) – risk factors in the development of TRD and MDpsy.

Interestingly, DBH knockout mice exhibited treatment resistance to some SSRIs when administered acutely (Willner & Belzung, 2015) raising a possible issue of too-short duration of treatments contributing to treatment resistance (Rosenzweig-Lipson *et al.*, 2007).

### 4.5 Norepinephrinergic and serotonergic dysfunction in MDpsy

Despite a hyperdopaminergic state and concurrent reduced levels of DBH, Goekoop *et al.* (2012) showed elevated plasma NE in MDpsy. This increased NE may be genetically determined (Keller *et al.*, 2007) or result from traumatic experience (Goekoop *et al.*, 2012). The latter correlates with previous work (Hess *et al.*, 2009) that showed that early childhood stress produced NE activation with an increased DBH activity followed by chronic suppression of DBH. Goekoop *et al.* (2012) showed that this increased release of NE is not state-dependent and may differentiate MDpsy from MD. MDpsy patients have higher CSF 5-hydroxyindoleacetic acid (HIAA) levels than MD patients (Schatzberg & Rothschild, 1992). Increased 5–HT platelet concentrations are associated with psychotic symptoms, cognitive disturbances, and aggression (Mück-Šeler *et al.*, 1996; Pivac *et al.*, 2006). The platelets of MDpsy patients exhibit higher 5-HT uptake rates as in MD patients and this difference is maintained after treatment but not after clinical recovery (Healy *et al.*, 1986). Thus elevated NE activity may be a trait marker (Goekoop *et al.*, 2012) while elevated 5-HT may be a state marker of MDpsy (Healy *et al.*, 1986).

### 4.6 Genetic risk

Several independent studies have suggested that MDpsy is primarily a consequence of genetics, having noted a higher risk of MDpsy in families of probands of MDpsy (Domschke, 2013). A registry-based twin study determined that MDpsy had a heritability of 39% (Lyons *et al.*, 1998) comparable to that of MD at 38% (Domschke, 2013).

Despite similar rates of heritability, TRD (including MDpsy) and treatment-responsive MD seem to have dissimilar genetic constitutions (Fabbri *et al.*, 2019). Genome-wide association studies (GWAS) revealed a number of potential genetic markers of TRD (Corvin *et al.*, 2010). These include: 1) glutamate (NDMA) receptor 2B subunit (GRIN2B), the increased expression of which increased the risk of developing TRD (Zhang *et al.*, 2014); 2) GRIK4 increases the risk of developing TRD and concurrent psychotic symptoms (Minelli *et al.*, 2016); 3) catechol-O-methyltransferase (COMT) which increases suicide in TRD (Schosser *et al.*, 2012).

GRIK4 (Glutamate Receptor Ionotropic Kainate 4) is predominantly expressed in the hippocampus (Fabbri *et al.*, 2019; Knight *et al.*, 2012) and amygdala (Schosser *et al.*, 2012). Variants of this gene

were previously associated with varying response to antidepressants (Milanesi *et al.*, 2015). One of the variants, the rs11218030 G allele, was linked to TRD and revealed significant associations with the presence of psychotic symptoms during depressive episodes (Milanesi *et al.*, 2015; Minelli *et al.*, 2016). Polymorphisms of the val66met allele of BDNF were also found to be associated with psychotic features and suicidal behaviour observed in depressive Japanese populations (Domschke, 2013).

## 5. Treatment of MD and MDpsy

The treatment of MD has primarily been focused on the regulation of the monoaminergic system. This is achieved mostly (not exclusively) through the use of monoamine reuptake inhibitors such as SSRIs and TCAs to inhibit the reuptake of serotonin (5-HT) norepinephrine (NE) and dopamine (DA) by serotonin transporters (SERT), norepinephrine transporters (NET), dopamine transporters (DAT), respectively; and by inhibiting the degradation of these monoamine neurotransmitters by monoamine oxidase (Hillhouse & Porter, 2015; López-Muñoz & Alamo, 2009). However, over time these antidepressants have proven to be less than ideal in treating MD because of their slow response times, numerous unfavourable side effects, and the possibility of relapse (Nestler *et al.*, 2002; Rosenzweig-Lipson *et al.*, 2007).

## 6. Treatment-resistance

The time course of depressive symptoms and its response to acute and chronic treatment was first proposed by Kupfer (1991) (Figure 2), and is of relevance here. Kupfer coined the terms "response", "remission", "recovery", "relapse", and "recurrence" as it occurs over time following treatment. Response is when the patient is no longer fully clinically symptomatic; that is, usually ≥50% reduction of symptoms (Frank *et al.*, 1991; Saltiel & Silvershein, 2015). Remission occurs when symptoms are absent or if the patient scores ≤7 (Frank *et al.*, 1991; Obeid *et al.*, 2018). Recovery can be defined as remission lasting for 2 or more months. Relapse is the return of symptoms between response and recovery (typically during remission) (Frank *et al.*, 1991). Recurrence is the development of a new MD episode following recovery (Frank *et al.*, 1991).

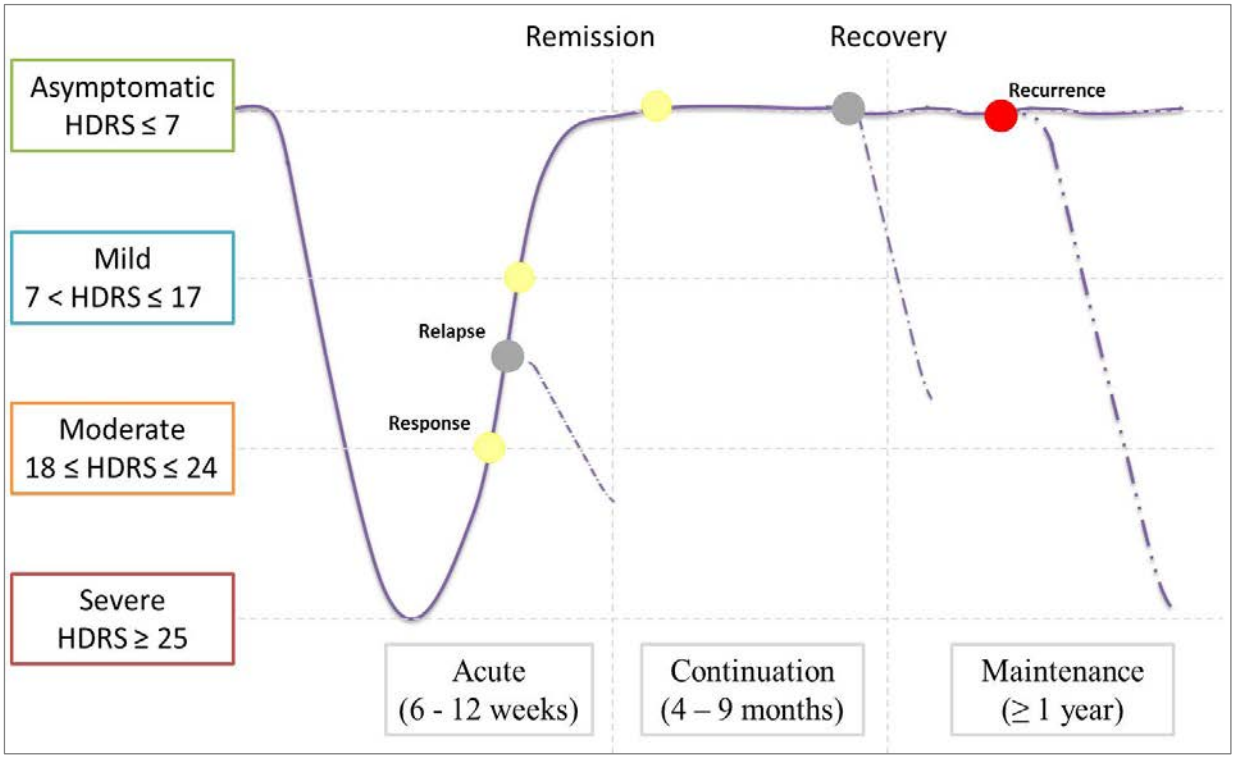


**Figure 2-2:** Time course of depressive symptoms showing response to acute and chronic treatment, relapse, recovery, and recurrence. Adapted from (Kupfer, 1991).

Of the estimated 216 million to 322 million people affected by MD worldwide (Cusin & Peyda, 2019), up to a third of these receiving treatment for MD will not respond to the first prescribed antidepressant (Berlim & Turecki, 2007). Clinical response to treatment is defined as a 50% or greater reduction in depressive symptoms (Hough *et al.*, 2017), while remission is reflected by a score of ≤7 on the 17-item Hamilton Depression Rating Scale (HDRS) (Rush *et al.*, 2004). The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study showed that after sequential administration of two antidepressant treatments, only 50 to 55% of depressive patients reached remission (Rush *et al.*, 2004), although (Hamon & Blier, 2013) states that 10 – 20% of patients still do not reach remission even after sequential medication. This failure to respond to the first or subsequent antidepressant treatment is termed treatment-resistant (or treatment-refractory) depression (Berlim & Turecki, 2007; Chaput *et al.*, 2008).

The "hit-or-miss" approach to treating depression has resulted in as many as 42% of patients failing to comply with their treatment regimen within the first 30 days of their first treatment if they feel no improvement in their symptoms (Leuchter *et al.*, 2010). Various risk factors for developing

TRD have been identified, amongst them are: depression subtypes (Berlim & Turecki, 2007), psychiatric comorbidity (Cepeda *et al.*, 2018), clinical factors (such as family history) (Kornstein & Schneider, 2001), premature cessation of treatment (Harvey & Slabbert, 2014), and biological markers (such as dexamethasone suppression) (Rothschild, 2013).

The treatment-resistant patient will continue to exhibit symptoms of severe depression and not achieve remission, or will relapse following either standard antidepressant pharmacotherapy at optimal doses or even rigorous pharmacological and psychotherapeutic interventions (Cusin & Peyda, 2019; Mathew, 2008; Rosenzweig-Lipson *et al.*, 2007; Warden *et al.*, 2007). MD is debilitating; however, TRD patients are at least twice as likely to be hospitalized (for both psychiatric and medical reasons), have a higher overall mortality from all causes, and are at much greater risk for suicide attempts (Cusin & Peyda, 2019). Furthermore, repeated episodes of depression results in further episodes (recurrence) that begin to cycle faster over time and which are often more severe and treatment resistant (Bschor *et al.*, 2014; Rizvi *et al.*, 2014). Such rapid cycling may also lead to mania as seen in BD (Akiskal *et al.*, 1983).

### 6.1 Psychosis: The difference between treatment responsiveness and resistance

A common basis for TRD is the presence of psychosis and ensuing prescription of inappropriate medication (Fink, 2003).

At present, no international consensus has been reached concerning the treatment of MDpsy. The South African Society of Psychiatrists recommends a combination of an antidepressant and antipsychotic as first-line therapy and electroconvulsive therapy (ECT) as second-line therapy (Rothschild, 2013), while other professional psychiatric bodies recommend using either the combination therapy or ECT as first-line treatment or even antidepressant monotherapy as first-line treatment (Rothschild, 2013). Current treatments of MDpsy are based on physicians' extrapolations of drugs developed for depression and psychosis, while no drug treatments have been specifically developed for psychotic depression (Rothschild, 2009).

A meta-analysis conducted by Farahani and Correll (2012) proved the efficacy of antidepressant monotherapy and even monotherapy with an antipsychotic to be inferior to co-treatment with an antipsychotic and an antidepressant. However, studies have also shown that only 5% of MDpsy

patients receive appropriate co-treatment for long enough periods and in adequate doses (Rothschild, 2013). This may, in part, be a result of failure to detect the psychotic aspect of the disorder (Rothschild, 2013). On the other hand, MDpsy patients are aware that their hallucinations are not "quite right" which may be accompanied by shame or fear of being institutionalised, thus preventing them from disclosing this symptom and compounding the problem of misdiagnosis and inappropriate treatment (Rothschild, 2013; Schatzberg, 2003).

Over the years, combinations of various antidepressants and antipsychotics have been used in the treatment of MDpsy leading to the current co-therapy of fluoxetine/olanzapine being touted as an effective combination, following case reports and at least two clinical trials in which the combination was compared to olanzapine monotherapy and placebo (Rothschild *et al.*, 2004; Schatzberg, 2003). Remarkably, this combination has also demonstrated superior efficacy for treating resistant MD compared to either agent alone (Shelton *et al.*, 2001). The olanzapine-fluoxetine combined formulation is currently approved by the FDA for TRD treatment (Caldarone *et al.*, 2015) but has been shown to be effective in treating MDpsy (Rothschild, 2013).

### 6.2 Putative mechanisms of action for fluoxetine/olanzapine combination

While antipsychotic monotherapy has been shown to be ineffective in treating MDpsy, at least two research groups have claimed olanzapine monotherapy to be effective in prompting substantial improvements in psychotic depressive symptoms (Shah *et al.*, 2016; Zhang *et al.*, 2000). This may be due to olanzapine's antagonistic effects on multiple dopamine, serotonin, and α-adrenergic receptors (Schatzberg, 2003) showing the highest affinity for 5-$HT_{2C}$ and $\alpha_1$-adrenergic (Zhang *et al.*, 2000). Olanzapine reduces the levels of IL-6 and TNF-α while increasing BDNF both of which may promote brain remodelling and neurogenesis, further contributing to its clinical efficacy (Hatziagelaki *et al.*, 2019). Fluoxetine has a relatively high affinity for 5-$HT_{2C}$ receptors and may modulate extracellular DA and NE transmission via excitatory effects on GABAergic interneurons (Bymaster *et al.*, 2002; Zhang *et al.*, 2000). The combination of olanzapine and fluoxetine was shown to result in sustained increases in extracellular levels of DA and NE and a sustained increase in 5-HT in the prefrontal cortices of rats (Zhang *et al.*, 2000). Clozapine and fluoxetine as well as risperidone and fluoxetine both raised extracellular NE and 5-HT but not significantly so compared to fluoxetine alone (Zou *et al.*, 2010). Olanzapine and fluoxetine produced a sustained release of extracellular DA to a greater extent than olanzapine combined with sertraline (Shah *et al.*, 2016;

Zhang *et al.*, 2000). Zhang *et al.* (2000) suggests this may be mediated by either 5-$HT_{2C}$ or $\alpha_1$-adrenergic receptors although 5-$HT_7$ has also been proposed (in TRD at least) (Wang *et al.*, 2016). Furthermore, these two drugs have been shown to up-regulate transcription factors that elicit sustained changes in synaptic efficacy and ultimately cognitive behaviour (Horowitz *et al.*, 2003; Zhang *et al.*, 2000).

## 7. Bipolar disorder (BD): risk factor for and relation to MDpsy

Patients diagnosed with MDpsy are at a high risk of developing bipolar disorder (Ostergaard *et al.*, 2014b). Both disorders share some physiological aberrations discussed below.

A feature of MDpsy is a family history of mental disorder, particularly BD type I (Keller *et al.*, 2007). The relationship between BD and MDpsy was initially suggested by various clinical studies, one of which showed a six times greater likelihood of psychotically depressed patients having a bipolar relative compared to MD patients and healthy controls (Weissman *et al.*, 1984). Nearly twice as many psychotically depressed patients had a history of mania compared to MD patients (Coryell *et al.*, 1984). Two other studies showed MDpsy patients ranging from adolescents to adults were more likely to be bipolar at follow-up than MD patients (Akiskal *et al.*, 1983; Strober & Carlson, 1982).

BD is a progressive and complex mood disorder characterised by erratic cycles of mania, depression, and mixed mood with periods of total or relatively symptom-free recovery (euthymia) (Cosgrove *et al.*, 2016; Harrison, 2016). Distinct manic episodes are required for the diagnosis of BD – the duration and severity of which differentiate BD type I from BD type II (APA, 2013). Manic episodes are characterised by symptoms such as an abnormally elevated mood, decreased need for sleep, extensive involvement in pleasurable activities, hyperactivity, psychomotor agitation, aggression, being easily distracted, and increased risk-taking behaviour. Depressive episodes are characterised by feelings of sadness, guilt, persistent lethargy, anhedonia, and eating disturbances resulting in weight gain or weight loss (APA, 2013). Impairment of neurocognitive and psychosocial functioning is also observed in BD (Gómez-Benito *et al.*, 2020).

The clinical manifestations of BD are complex and can change drastically over time, complicating clinical management of this disorder. Lithium has been a fundamental treatment for BD for many

decades, although more recently anticonvulsants and second generation antipsychotics have been added to the BD treatment arsenal (Baldessarini *et al.*, 2019; Young *et al.*, 2011). Antipsychotic drugs are also used as monotherapy to effectively treat mania; however, the treatments seem to exert symptom-specific effects (Young *et al.*, 2011). In particular, a reduction in hostility is seen with risperidone and aripripazole treatment while olanzapine improves elevated mood; quetiapine results in overall improvement (Young *et al.*, 2011). Even so, Gitlin *et al.* (1995) estimated patient relapse into affective episodes at 37% within a year of treatment. This increases to nearly to 60% after 2 years and to 75% after 5 years (Alafchi *et al.*, 2018). Relapses lead to neuroprogression, evidenced by shortened intervals between mood cycles; increased likelihood of co-morbidity; treatment resistance to pharmacotherapy and psychotherapy; functional and structural brain modifications; as well as diminished cognitive function (Berk *et al.*, 2011; da Costa *et al.*, 2016; Gama *et al.*, 2013; Kessing *et al.*, 2011).

BD is associated with chronic low-grade inflammation. Elevated levels of the pro-inflammatory cytokines, IL-6 and TNF-α, are observed throughout the early and late stages of BD with TNF-α further increasing in the latter stage of the disorder (da Costa *et al.*, 2016; Post *et al.*, 2012; SayuriYamagata *et al.*, 2017). A meta-analysis of 30 studies comparing blood cytokine concentrations of healthy subjects vs. BD patients found marked elevations of TNFα, soluble TNF-α receptor (sTNF-R1), IL-4, IL-6, soluble IL-6 receptor (sIL-6R), IL-10, IL-1 receptor antagonist (IL-1RA), and soluble IL-2 receptor (sIL-2R) (Modabbernia *et al.*, 2013). Elevated TNF-α and sTNF-R1 were observed in manic patients compared to their euthymic counterparts (da Costa *et al.*, 2016), confirming the influence of inflammatory mediators on mood (Modabbernia *et al.*, 2013; SayuriYamagata *et al.*, 2017).

HPA-axis hyperactivity and resultant high cortisol levels are observed in BD most prominently in the manic phase, but also occurring in remission (Muneer, 2016). HPA-axis dysfunction (most notably hypercortisolism) erodes psychobiological resilience and coping leading to increased vulnerability to recurrent episodes and illness progression (Muneer, 2016), and may be central to the development of depressive symptoms and cognitive deficits (Sigitova *et al.*, 2017). A hyperdopaminergic state in BD is associated with mania (Amann & Grunze, 2005) similar to the association between hyperdopaminergia and psychosis in MDpsy. Reduced plasma DBH is associated with MDpsy while elevations are observed in psychotic BD (Hamner, 2011). DBH does

not differ significantly between non-psychotic BD patients compared to healthy controls (Hess *et al.*, 2009). Decreased 5-HT is associated with the depressive and manic phase of BD (Mahmood & Silverstone, 2001) and is likely caused by the inflammation-mediated activation of IDO driving tryptophan metabolism to quinolinic acid and depleting the neuronal pool of 5-HT (Sigitova *et al.*, 2017). This is also noted in MDpsy (discussed in section 1.4.2). The involvement of norepinephrine in BD is linked to the phase of the disorder where increased urinary levels of norepinephrine and its metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG) are associated with mania (and switching to mania) but not depression (Kurita, 2016). Instead reduced MHPG levels were reported to be a biomarker for the transition from mania to remission (Kurita *et al.*, 2015).

These biochemical deviations: immune activation and oxidative stress, disordered dopaminergic and neutrophin systems, HPA-axis hyperactivity and deficient neuroprotection contribute to neuroprogression of the illness (Berk *et al.*, 2011; da Costa *et al.*, 2016; Kato, 2017).

As noted earlier, lithium is the treatment of choice for BD (Baldessarini *et al.*, 2019; Young *et al.*, 2011). Lithium appears to preserve or increase the emotional brain structures including the prefrontal cortex and amygdala, and upregulates hippocampal neurogenesis (Jope, 1999; Kin *et al.*, 2019). Lithium reduces dopamine and glutamate, enhances cholinergic activity, and increases inhibitory (GABA) neurotransmission (Jope, 1999; Malhi *et al.*, 2013). Lithium has also been reported to stabilise serotonergic neurotransmission (Jope, 1999) by reducing serotonin binding in the hippocampus but not in the cortex (Treiser *et al.*, 1981). Lithium also possesses anti-suicidal properties (Malhi *et al.*, 2013). Furthermore, lithium was reported to counteract depressive behaviour in a chronic mild stress rat model and also showed efficacy in the Wistar Kyoto (WKY) rat model of TRD, which typically does not respond to fluoxetine (Kin *et al.*, 2019). Clinically, when administered with another antidepressant, lithium induces rapid relief of depression in drug non-responders (Kin *et al.*, 2019). In the aforementioned WKY study, lithium was co-administered with fluoxetine resulting in a pronounced antidepressant response (Kin *et al.*, 2019). Considering the elevated dopamine and serotonin levels present in MDpsy, hippocampal atrophy as well as the significant suicide rate, lithium may also be useful in the treatment of MDpsy. Lithium exerts anti-inflammatory effects in BD patients by suppressing the production of pro-inflammatory cytokines, IL-6, TNF-α, and IL-β and increasing the production of the anti-inflammatory cytokine, IL-10

(Nassar & Azab, 2014). Lithium has been reported to normalise DBH activity (Paclt *et al.*, 2009) and to decrease HPA-axis activity (Machado-Vieira, 2018) in BD.

## 8. Modelling mood and psychiatric disorders in animals

Animal models are developed to capture, as closely as possible, the behavioural, neurochemical and physiological aspects of conditions occurring in humans so as to allow for invasive analysis of underlying mechanisms and possible treatments (Fuchs & Flugge, 2006; Hollis & Kabbaj, 2014). Given the shortfalls in our understanding of the biology and pharmacology of MDpsy as noted earlier, a need exists for a valid animal model of MDpsy to provide insight into the pathophysiology of the disorder, provide a platform to develop new treatment strategies, and to develop objective diagnostic criteria. The validity of an animal model relies on it meeting three principle criteria regarding: (1) behavioural changes analogous to the human condition – face validity; (2) reproduction of the pathophysiology of the disorder – construct validity; and (3) animal response to pharmacological treatments effective in humans – predictive validity (Fuchs & Flugge, 2006; Logan & McClung, 2016; Valvassori et al., 2013).

### 8.1 Modelling MD: The Flinder's Sensitive Line (FSL) rat

The FSL rat is widely described as a validated genetic animal model of MD that mimics the depressive disorder at various levels of expression (Overstreet & Wegener, 2013). The FSL rat was originally developed to be genetically resistant to the effects of the anticholinesterase agent, di-isopropyl fluorophosphates (DFP), to elucidate the mechanism involved in the development of resistance to DFP (Overstreet *et al.*, 2005). Instead, a strain that is genetically more sensitive to DFP was produced (Overstreet *et al.*, 2005). However, it was also later observed that these rats not only became increasingly sensitive to this cholinergic agent but also displayed behavioural characteristics akin to depressed humans. Indeed a cholinergic hypothesis of depression had been put forward in the early 1970's by Janowsky *et al.* (1972) which has gathered momentum over the past decades (Dulawa & Janowsky, 2019; Jeon *et al.*, 2015; Pytka *et al.*, 2016). Interestingly the counterpart of the FSL rat, the Flinders Resistant Line (FRL) rat, did not develop the intended cholinergic resistance but also did not show the characteristic depressive-phenotype of FSL rats (Overstreet *et al.*, 1998). Furthermore, with MD linked to decreased stress resilience as well as exposure to prior and on-going psychosocial stress, FSL rats are similarly highly stress-sensitive

(Brand & Harvey, 2017a, 2017b; Overstreet & Wegener, 2013). These attributes have established it as a useful animal model of MD.

The FSL rat has good face, construct, and predictive validity for MD (Overstreet *et al.*, 2005; Overstreet & Wegener, 2013). With respect to face validity, FSL rats display symptoms akin to depressed humans insofar as exhibiting increased REM sleep, reduced body weight (they generally weigh less than their resistant counterparts), cognitive (memory and learning) disruptions, and psychomotor retardation (Overstreet, 1993; Overstreet *et al.*, 2005). Curiously, anhedonia – a core symptom of MD has not been conclusively demonstrated in FSL rats although they do tend to be more anhedonic than the FRL rats when exposed to chronic mild stress (APA, 2013; Overstreet *et al.*, 2005). The stress-sensitive phenotype of the FSL rat has been demonstrated as increased anxiety (Liebenberg *et al.*, 2012), albeit only shown in certain behavioural tests of anxiety and not others (Overstreet & Wegener, 2013). FSL rats exhibit despair/hopelessness, cardinal behavioural symptoms of MD as measured using the forced swim test (FST) during which they demonstrate exaggerated immobility (APA, 2013; Overstreet, 1993). At the biological level FSL rats resemble depression-associated abnormalities in mitochondrial, redox and immune function (Oberholzer *et al.*, 2018) and alterations in monoaminergic and glutamatergic activity (Nishi *et al.*, 2009; Overstreet & Wegener, 2013). Furthermore, as was previously mentioned, the FSL rats are more sensitive to cholinergic agents (Overstreet, 1993) likely due to the greater number of muscarinic receptors in several brain regions (Overstreet *et al.*, 2005). FSL rats also present with phase advanced circadian rhythms (Overstreet *et al.*, 2005). The increased susceptibility of FSL rats to stress has been linked to increased activation of the nitric oxide synthase (NOS) cascade with increased production of nitric oxide (NO) (Wegener *et al.*, 2010). The NO cascade is implicated in the immune-inflammatory response (Roomruangwong *et al.*, 2018), as well as to interact with serotonergic and cholinergic function, all of which, when altered, are implicated in depression and schizophrenia (Brand *et al.*, 2015) and in treatment resistance (Harvey, 1996; Harvey *et al.*, 2003).

Finally, well-recognized antidepressant drugs such as imipramine, desipramine, and sertraline successfully reverse the above-mentioned exaggerated depressive-like behaviour, especially immobility, in FSL rats. Importantly, this response is noted predominately following chronic and not acute treatment, thus supporting the predictive validity of the model (Overstreet, 1993; Overstreet & Wegener, 2013).

### 8.1.1 Measuring depressive-like behaviour: The Forced Swim Test (FST)

The forced swim test (FST) assesses antidepressant activity by measuring despair-like behaviour (Cryan *et al.*, 2002; Porsolt *et al.*, 1977), characteristic of depression (APA, 2013).

The rat, when placed in a cylinder filled with water, will initially make attempts to escape the aversive situation by "climbing" in an upward direction along the side of the cylinder or "swimming" throughout the cylinder (Cryan *et al.*, 2002). These escape-directed behaviours are related to inherent coping strategies initiated to deal with and overcome an adverse environmental condition. Despair is expressed as diminished escape-oriented behaviour ultimately resulting in floating with just the animal's nose above water (immobility or inactivity) (Cryan *et al.*, 2002; Mokoena *et al.*, 2015). The traditional FST as developed by Porsolt *et al.* (1977), showed that antidepressant treatment prolonged the escape attempts of the rat, although did not reliably detect the effects of SSRIs. This shortcoming prompted the development of the modified FST (Cryan *et al.*, 2002); specifically, the water depth was increased allowing for various behaviours to be discriminated *viz.* climbing, swimming, and immobility as well as how these behaviours respond to various antidepressant treatments i.e.: NE re-uptake inhibitors (NRIs) increase climbing behaviour while SSRIs increase swimming behaviour (Cryan *et al.*, 2002).

## 8.2 Modelling psychosis: Social isolation rearing (SIR)

Psychosis is characterised by a distortion of reality (Forrest *et al.*, 2014) and may present with one or a combination of the following symptoms: hallucinations and/or delusions, agitated body movements, hyperlocomotor activity, disordered thoughts and behaviour, anxiety, or impaired social cognition (flat affect, aggression, suspiciousness) (APA, 2013; Forrest *et al.*, 2014).

Although neurobiologically, the DA hypothesis has developed into the most well-researched and well-supported model underlying the behavioural changes evident in schizophrenia, subsequent retrospective studies have noted that early life adversity, be it pre- or post-natal, is a prominent contributing factor in the neurodevelopment of the disorder later in life (Kim *et al.*, 2015; Weinstein *et al.*, 2017). Such early life stressors include childhood sexual abuse, physical abuse, and neglect as well as foetal exposure to hypoxia, mothers smoking during pregnancy, and prenatal maternal stress including loss of a spouse and mild infection (Kim *et al.*, 2015; Wickham & Bentall, 2016).

The rat colony and its defined social structure contribute to the normal development of a rat. Post-weaning social isolation rearing (SIR) disrupts this development by removing the young rat from its colony during early life and serves as a stressor that induces numerous late-life neurochemical, neurobiological and behavioural deviations that have been characterised as being related to psychosis-like behaviours (Forrest *et al.*, 2014; Heidbreder *et al.*, 2000; Jones *et al.*, 2011; Kim & Kirkpatrick, 1996; Moller *et al.*, 2015).

SIR of rats meets the validation criteria for an animal of psychosis in the following ways:

*Face validity*. SIR rats show signs of increased aggression and spontaneous locomotor hyperactivity in response to novel settings; deficits in sensorimotor gating and novel object recognition; and heightened anxiety (Ko *et al.*, 2016; Moller *et al.*, 2015). SIR rats also display increased self-directed and decreased social interactive behaviours (Moller *et al.*, 2015) as well as depressive-like behaviours (i.e. decreased mobility in the FST) (Vargas *et al.*, 2016), thus indicative of negative symptoms.

*Construct validity*. Post-weaning isolation rearing has been shown to alter signal transduction of transmitters whose dysfunction has been implicated in psychosis and schizophrenia including: changes in cortico-striatal glutamate, DA, and 5-HT levels; potentiation of brain ACTH; a pro-inflammatory state (Fone & Porkess, 2008; Forrest *et al.*, 2014; Jones *et al.*, 2011; Kim & Kirkpatrick, 1996; Moller *et al.*, 2015); evidence for oxidative stress and mitochondrial dysfunction (Moller *et al.*, 2011; Moller *et al.*, 2013; Mumtaz *et al.*, 2018), disordered tryptophan-kynurenine metabolism (Möller *et al.*, 2012), and altered oxytocin/vasopressin levels (Harvey *et al.*, 2019).

Predictive validity. Psychosis-like behavioural and neurochemical changes, including anxiety and cognitive deficits, are responsive to anxiolytic, antidepressant and antipsychotic drug treatment (Moller *et al.*, 2013; Regenass *et al.*, 2018; Toua *et al.*, 2010). It also shows response to the antioxidant, N-acetyl cysteine (NAC) (Moller *et al.*, 2013).

### 8.2.1 Measuring psychosis-like behaviour: Prepulse Inhibition (PPI) test

PPI - observed in normal subjects (humans and animals) - refers to the normal reduction in startle magnitude occurring when an intense startling stimulus is immediately preceded by a weak pre-stimulus (Castagne *et al.*, 2009; Macedo *et al.*, 2012). PPI is a measure of sensorimotor gating which is the ability to filter irrelevant and excessive incoming information in favour of focusing attention on processing more important stimuli (Castagne *et al.*, 2009; Shoji & Miyakawa, 2018). This sensory filtering by neural circuits prevents cognitive fragmentation (Ahmari *et al.*, 2012; Braff & Geyer, 1990). PPI deficits observed in schizophrenic patients correlate to clinical symptoms of disordered thoughts and distractibility (Forrest *et al.*, 2014). Because of its translational validity to animals (Braff *et al.*, 2001; Varty *et al.*, 2000), it is a useful tool for investigating psychosis-like symptoms in rodents (mice, rats, and guinea pigs). Unlike the blink reflex in humans, PPI is measured in rodents as a whole-body startle elicited by an acoustic startle-eliciting stimulus (Jones *et al.*, 2011; Shoji & Miyakawa, 2018).

PPI is regulated by the cortical (medial prefrontal cortex) and subcortical structures (striatum) (McCutcheon *et al.*, 2019; Swerdlow, 2009). PPI deficits are associated with DA alterations, such as those resulting from amphetamine or apomorphine administration and SIR, and are typically reversible with appropriate antipsychotic drug treatment (Castagne *et al.*, 2009; Forrest *et al.*, 2014). In our environment, PPI of acoustic startle is an effective behavioural measure reflecting cognitive fragmentation in rodents and its response to novel treatments (Uys *et al.*, 2016).

## 9. Modelling treatment-resistant depression

There is currently a scarcity of suitable animal models of TRD which has hindered scientific investigation into the genetic and neurobiological abnormalities that underlie resistance to traditional antidepressants. Some approaches that have been used to develop rodent models of TRD have included: 1) exposure to chronic mild stress or chronic social defeat (Samuels *et al.*, 2011); 2) treating animals with agents that render them resistant to antidepressant therapy, for example, pro-inflammatory cytokines (Stepanichev *et al.*, 2014; Sukoff Rizzo *et al.*, 2012) and cortisol (Kitamura *et al.*, 2002; Pereira *et al.*) or ACTH (Pereira et al., 2019); 3) maternal separation in FSL rats (Piubelli *et al.*, 2011); and 4) exposing FSL rats to a PTSD paradigm such as time-dependent sensitisation (TDS; Brand and Harvey, 2017a; 2017b). These models, however, do not address the

issue of psychosis occurring in major depression with psychotic features as well as its cyclical nature. The closest animal model to MDpsy, at least in the co-occurrence of depression (manifested as hypo-activity) and mania (demonstrated by hyperactivity), is the ouabain model for bipolar disorder (El-Mallakh *et al.*, 1995). However, it lacks other features of MDpsy such as genetic or severe environmental stress that predispose some humans to develop depression and co-occurring psychosis. Furthermore, features such as psychomotor retardation, social impairment, despair-like behaviour, and sensorimotor gating deficits have not been shown in this model (review Valvassori *et al.* (2019)).

While no single animal model can fully represent the entirety of the MDpsy syndrome, it is important that it resemble the disorder as closely as possible in symptomology, biology and response to pharmacotherapy to better inform of effective therapies and potential diagnostic markers. Phenomenology is also important, where the construct surrounding the development of the animal model is close to that observed in humans. In this regard, subjecting a genetically susceptible animal to a PTSD paradigm may induce a pattern of TRD in the animal later in life (Brand & Harvey, 2017a, 2017b). Similarly, since early life adversity, inherent high levels of anxiety and stress-sensitivity are recognised as being important neurodevelopmental factors in the later development of MDpsy and TRD (Cepeda *et al.*, 2018; Keller *et al.*, 2007; Nasca *et al.*, 2018), combining a neurodevelopmental model of schizophrenia like social isolation rearing (SIR) with a genetic model of depression (FSL rats) may offer similar capabilities, but with the added incentive of providing enhanced psychosis-like behaviours akin to MDpsy.

Animal models of depression have largely focused on determining antidepressant efficacy (Caldarone *et al.*, 2015). Caldarone *et al.* (2015) states that animal models of TRD should exhibit non-response to traditional treatments as well as antidepressant-like responses to treatments effective in similarly resistant populations. Willner and Belzung (2015) argues that since TRD is typically associated with risk factors that predispose an individual to developing depressive episodes, animals models should incorporate factors that predispose to TRD-like behaviour in addition to being less responsive to traditional antidepressant treatment, e.g.: heightened stress responsiveness and disordered HPA-axis activity. Using these suggestions as a guide, an animal model of MDpsy ideally should include risk factors for TRD (for example, propensity to develop depression) and psychosis as well as show poor response to traditional antidepressant treatment.

Finally, because of the close association between BD and MDpsy, i.e. neurodevelopmentally, biologically, behaviourally and with respect to drug treatment, it is possible that an effective MDpsy model may offer some utility as a model of BD.

# CHAPTER 3: MANUSCRIPT A

**Preamble**

This chapter presents the full-length manuscript titled: **Flinders Sensitive Line rats exposed to post weaning isolation rearing are an animal model of treatment resistant depression** and submitted to the *Journal of Affective Disorders* published by Elsevier. Instructions to the author regarding online submission and formatting requirements of manuscripts are available at: https://www.elsevier.com/journals/journal-of-affective-disorders/0165-0327/guide-for-authors. The requirements for formatting were strictly adhered to for the online submission, however, for the sake of ease of readability the format and layout of the manuscript has been slightly amended to comply with the rest of the thesis. Thus, the figure legends and tables are inserted into the text with their corresponding paragraphs rather than after the references as stipulated in the author guidelines of this journal.

According to research Question 1 (see *Research questions* in Chapter 1 section 3.2), this manuscript reports the development and bio-behavioural validation of a tentative animal model of treatment-resistant depression (TRD) that follows exposing a genetic rodent model of depression, the FSL rat, to post weaning social isolation rearing (SIR) (see *Figure 1: Study design* in this Manuscript), a possible dual-hit model of TRD. This manuscript reports the TRD-related bio-behavioural changes occurring in the model and evaluates the model's response to the antidepressant, FLX. The FSL-SIR model is compared to SAL-treated SD control rats to establish baseline bio-behavioural anomalies, and to compare these changes to those presenting in SAL-treated FSL rats. The latter is to show similarities to and differences from treatment responsive depressive-like manifestations. FLX-treated FSL-SIR rats are compared to SAL-treated SD and FSL rats as well as FLX-treated FSL rats to determine the extent of reversal or resistance of the presenting bio-behavioural parameters to standard antidepressant treatment relative to these controls. Since the hypothesis here relates to the effect of rearing condition on the development of TRD-like anomalies and the effect of treatment on the same anomalies, a two-way ANOVA is used to analyse interaction between treatment and rearing condition.

# Chapter 3: Manuscript A

**Both co-authors have provided permission to submit this manuscript for examination as part of K Mncube's Ph.D thesis. The letters of consent are included in Addendum D.**

# Chapter 3: Manuscript A

## Flinders Sensitive Line rats exposed to post weaning isolation rearing are an animal model of treatment resistant depression

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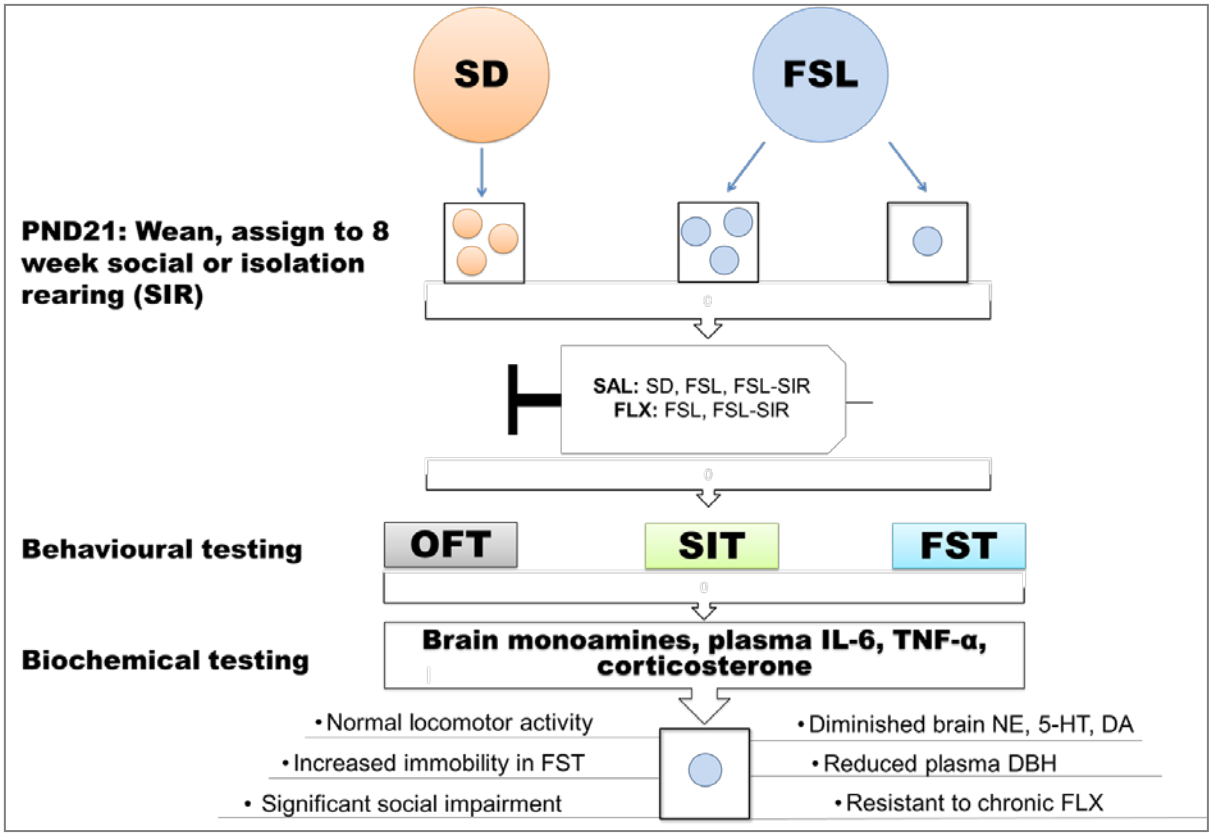
# Chapter 3: Manuscript A

## Highlights

- A novel animal model of treatment resistant depression (TRD)
- Displays exacerbated depressive-like behaviours following chronic fluoxetine (FLX)
- Displays social impairments unresponsive to chronic FLX treatment
- Displays depleted cortico-hippocampal monoamines variably reversed by chronic FLX
- Exhibits depleted DA and reduced plasma DBH partially reversed by FLX treatment

## Graphical abstract



# Chapter 3: Manuscript A


## Abstract

### Background

Treatment resistant depression (TRD) complicates the management of depression (MD). Its underlying biology involves interplay between genetic propensity and chronic and/or early life adversity. By combining a genetic animal model of MD and post-weaning social isolation rearing (SIR), we sought to produce an animal that displays more severe depressive- and social anxiety-like manifestations resistant to standard antidepressant treatment.

### Methods

Flinders Sensitive Line (FSL) pups were reared in isolation or socially from weaning (postnatal day (PND) 21), receiving fluoxetine (FLX) from PND63 (10 mg/kg x 14 days), and compared to Sprague Dawley (SD) controls. Depressive-, anxiety-like, and social behaviour were assessed from PND72. Post-mortem cortico-hippocampal norepinephrine (NE), serotonin (5-HT), and dopamine (DA), as well as plasma interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α), corticosterone (CORT), and dopamine-beta-hydroxylase (DBH) levels were assayed.

### Results

FSL rats displayed disturbed cortico-hippocampal monoamines and depressive- and social anxiety-like behaviour, the latter responsive to FLX. SIR-exposed FSL rats exhibited significant immobility in the FST and social impairment worsened by or resistant to FLX. In SIR-exposed FSL rats, FLX significantly reversed depleted NE and 5-HT, significantly decreased DBH and caused a large effect size increase in DA and decrease in CORT and TNF-α.

### Limitations

SIR may have introduced psychotic-like symptoms not assessed or treated in this study.

### Conclusion

SIR-exposed FSL rats display depressive- and social anxiety-like symptoms that are resistant to, or worsened by, FLX, with reduced plasma DBH and suppressed cortico-hippocampal 5-HT, NE and DA, all variably reversed by FLX.

**Keywords:** treatment-resistant depression; gene-X-environment model; fluoxetine; animal model; social anxiety; forced swim test.

## 1. Introduction[2]

While major depression (MD) affects approximately 216-322 million people worldwide (Cusin & Peyda, 2019), up to a third are non-responsive to an antidepressant (Berlim & Turecki, 2007) with up to a half failing to reach remission (Trivedi *et al.*, 2006). In such cases of treatment resistant depression (TRD), approximately 30% remain non-responsive to treatment after several treatment interventions (Al-Harbi, 2012) with that number decreasing with subsequent trials (Rush *et al.*, 2006).

The factors contributing towards the development of TRD include 1) late age of onset of MD and family history (Kornstein & Schneider, 2001); 2) non-response to first antidepressant (Rosenzweig-Lipson *et al.*, 2007); 3) history of abuse (sexual, physical, neglect) (Nasca *et al.*, 2018); 4) personality traits (Takahashi *et al.*, 2013) and personality disorder (Souery *et al.*, 2007); 5) comorbid psychiatric disorders (especially anxiety), insomnia, pain sensitivity, and gender (Cepeda *et al.*, 2018); 6) current risk of suicide (Rosenzweig-Lipson *et al.*, 2007); 7) high recurrence rates (Rizvi *et al.*, 2014); 8) and undetected psychotic symptoms (Fink, 2003). The treatment of MD has traditionally targeted monoaminergic systems by either blocking monoamine degradation or monoamine reuptake sites (Bobo & Shelton, 2009). Fluoxetine (FLX) is a selective serotonin reuptake inhibitor (SSRI) widely regarded as a standard-of-care treatment for MD and various anxiety disorders (Hurst & Lamb, 2000). Mechanistically, FLX increases 5-HT while effecting moderate increases in frontocortical and hypothalamic norepinephrine (NE) and dopamine (DA) (Bymaster *et al.*, 2002).

Compared to MD patients, TRD has been associated with decreased dopamine-β-hydroxylase (DBH) (Caldarone *et al.*, 2015), as well as hypothalamic-pituitary-adrenal (HPA)-axis hyperactivity,

[2] Abbreviations: MD, depression; TRD, treatment-resistant depression; SD, Sprague Dawley; FSL, Flinders Sensitive Line; PND, postnatal day; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine; OFT, open field test; SIT, social interaction test; FST, forced swim test; NE, norepinephrine; 5-HT, serotonin; SERT, serotonin transporters, DA, dopamine; DBH, dopamine-beta-hydroxylase (dopamine-beta-monooxygenase); CORT, corticosterone; HPA-axis, hypothalamic-pituitary-adrenal axis; IL-6, interleukin-6; TNF-α, tumour necrosis factor-α.

abrogated negative feedback and hypercortisolemia (Atmore *et al.*, 2020). Pro-inflammatory cytokines, interleukin (IL) -6 and tumour necrosis factor (TNF) –α, are also strongly implicated in the pathogenesis of MD, as well as being indicators and predictors of TRD (Brand *et al.*, 2015; Maes *et al.*, 1997). Concerning the monoamines, reduced dopamine (DA) neurotransmission is especially implicated in TRD (Hori & Kunugi, 2012; Wijeratne & Sachdev, 2008), although elevated DA in cerebrospinal fluid and plasma is typically observed in TRD with psychotic features (Schatzberg & Rothschild, 1992). Animal models have similarly noted depleted prefrontocortical DA (Caldarone *et al.*, 2015), as well as increased mesolimbic ventral tegmental dopaminergic activity (Caraci *et al.*, 2018). Serotonin deficits in fronto-limbic sites (Coplan *et al.*, 2014) and inefficient NE neurotransmission is also associated with TRD (Maletic *et al.*, 2017). Neuroanatomically, frontocortical dysfunction is postulated to underlie much of the cognitive and negative affect evident in MD, including anxiety and social deficits, while deficits in cognition, affect and volition are typically of hippocampal origin (Uys *et al.*, 2017).

The development of MD is influenced by genetic susceptibility as well as adverse environmental factors (Villas Boas *et al.*, 2019). The Flinders Sensitive Line (FSL) rat displays behaviour akin to depressed humans, and is a useful genetic animal model with broad face, construct, and predictive validity for MD (Overstreet, 1993; Overstreet & Wegener, 2013). Its shows broad response to various antidepressants following chronic dosing (Overstreet & Wegener, 2013). On the other hand, childhood adversity is known to be prodromal in the development of MD and TRD (Nasca *et al.*, 2018; Willner & Belzung, 2015). In animal studies, removing young rodents from their colony at weaning induces long-lasting behavioural changes that include neophobia, social withdrawal, disordered social interaction, and aggression (Fone & Porkess, 2008). In this regard, post-weaning social isolation rearing (SIR) in rodents models early-life neurodevelopmental changes that parallel the development of MD (Fone & Porkess, 2008), anxiety (Regenass *et al.*, 2018) and schizophrenia (psychosis) (Moller *et al.*, 2011).

Current animal models of TRD explore constructs such as a hyper-responsive HPA-axis (Pereira *et al.*, 2019), gene-x-stress/environment models (Brand & Harvey, 2017a), the Wistar Kyoto rat model (Kin *et al.*, 2019), and maternal separation (Marchetti *et al.*, 2020). Importantly, multiple pre- and post-natal adverse events are critical in the genesis of anxiety related and psychiatric disorders (Moller *et al.*, 2011; Swanepoel *et al.*, 2018). Considering TRD, clinical studies have shown an

association between treatment resistance and prior exposure to two or more adverse events (Nasca *et al.*, 2018), while a recent animal study has demonstrated that exposing FSL rats to severe stress with reminders not only amplifies depressive-like behaviour, but shows resistance to the tricyclic antidepressant, imipramine (Brand & Harvey, 2017a). Thus, current thinking would suggest that a second post-natal hit interacts with pre-existing genetic factors to trigger or aggravate behavioural symptoms that eventually drive the development of treatment-resistance (Brand & Harvey, 2017a; Vargas *et al.*, 2016).

Based on the above premise, this study aimed to develop a gene-x-environmental model of TRD by exposing FSL rats to post-weaning SIR and to evaluate the effect of treatment on the resulting model. We hypothesised that a more severe depressive-like profile would ensue with more pronounced social impairments, both showing resistance to FLX treatment. Further, we hypothesised that biochemical changes commensurate with TRD would co-present with treatment resistance, including reduced cortical and hippocampal monoamines, reduced plasma DBH as well as elevated plasma IL-6, TNF-α, and corticosterone (CORT).

## 2. Methods

### 2.1 Animals

This study was approved by the AnimCare animal research committee (NHREC reg. no. AREC-130913-015) of the North West University (NWU) (Ethics approval number: NWU-00150-18-S5). All animals used were bred, supplied and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform (PCDDP) at the NWU.

Since FSL rats are derived from the Sprague-Dawley (SD-SAL) strain, either SD-SAL or Flinders Resistant Line (FRL) rats are used as control animals (Overstreet & Wegener, 2013). The original colonies of FSL rats were obtained from Dr David H Overstreet, University of North Carolina, USA. The effects of SIR on anxiety and hyperactivity are not consistently observed in female rats (Walker *et al.*, 2019; Weiss *et al.*, 2004). Since this is a requirement for the proposed model, female rats were excluded from this study. All rats were allowed free access to standard laboratory chow and

water, and housed in identical transparent cages (380 mm x 380 mm x 230 mm) in an environmentally-controlled room: constant temperature (22 ± 4°C), humidity (50 ± 20%), and a 12:12 hour light-dark cycle (lights on 06:00) with no to minimal noise.

### 2.2 Study design

The study design is presented in Figure 1. Animals were weaned at post-natal day (PND) 21. All SD-SAL rats were assigned to social-rearing (3 rats/cage) while the FSL rats were randomly assigned to either social-rearing or social isolation rearing (SIR, 1 rat/cage). Rearing conditions were maintained for a period of 8 weeks (Moller *et al.*, 2013; Uys *et al.*, 2016). All animals were exposed to the same olfactory, visual, and auditory cues, although FSL-SIR rats were deprived of social contact with peer rats during this period. At PND 63, while remaining in their assigned rearing condition, FSL and FSL-SIR animals were assigned to a treatment group: either saline-treated (SAL) or FLX-treated (FLX). SD-SAL rats received only SAL. Thus, the resultant cohorts were as follows: SD-SAL, FSL-SAL, FSL-FLX, FSL-SIR-SAL, and FSL-SIR-FLX. Each cohort contained 12 rats (n = 12 per cohort) and a total of 60 animals were used in this study. The animals were first weighed on the day of weaning and then again each morning from the beginning of the treatment protocol (PND 63) until the last day of the study (PND 77). Their weights were used to calculate the volume of drug to be administered and to ensure equal growth in all the treatment groups. The treatment regimen commenced from PND 63 and continued until PND 76. Behavioural testing commenced on PND 72 beginning with the OFT, followed by the SIT on PND 74, and the FST on PND 75. This sequence orders the assessments from least to most stressful to ensure that the results of subsequent tests are not negatively affected by prior tests (Mokoena *et al.*, 2015). The animals were euthanised by decapitation without prior administration of an anaesthetic 24 h after the last behavioural test. Trunk blood and brain tissue were collected for bioanalysis. For behavioural and monoamine analysis, all animals (n = 12 per cohort) were included in the data. For ELISA analysis, ten (n = 10 per cohort) plasma samples were randomly selected from the 12 animals per cohort. This was to allow for more samples to be assayed per plate while maintaining statistical power. Quantification of these markers in plasma as opposed to in the brain is deliberately aimed at correlating to clinical findings which are mainly based on fluid sample readouts.

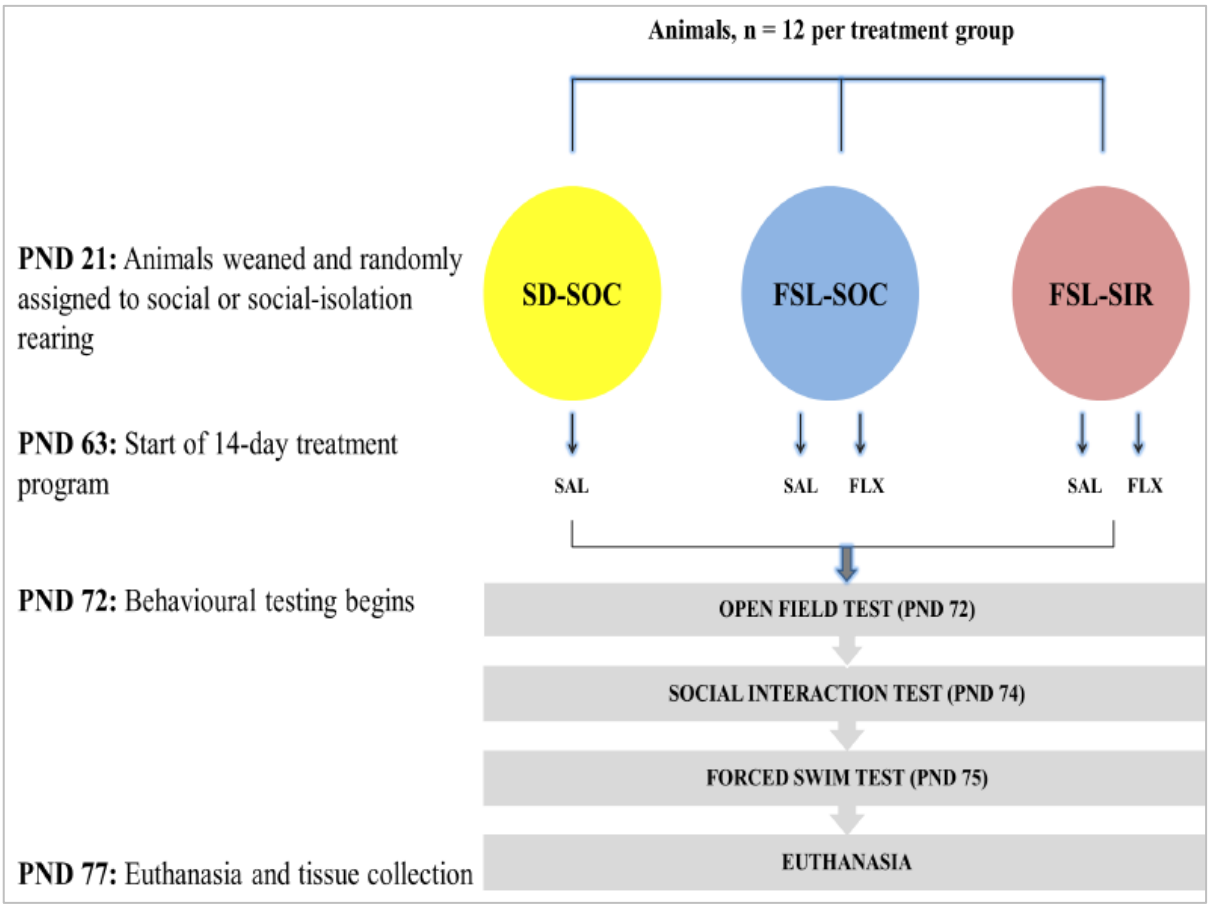



**Figure 1:** Study design

### 2.3 Drug preparation and treatment protocol

FLX (fluoxetine hydrochloride; Pubchem CID 62857) was a generous gift from Jade Pharmaceuticals, South Africa. FLX was first dissolved in approximately 500 µL distilled water and then made up to 10 mg/kg in physiological saline. FLX was administered at this dose as it was shown to most reliably affect swimming behaviour (a 5-HT mediated behaviour), reduce immobility, and to robustly increase extracellular 5-HT, NE, and DA (Detke *et al.*, 1995; Zhang *et al.*, 2000). FLX was administered subcutaneously (s.c.) for a period of 14 days. Control rats received SAL s.c. In both instances, treatment was administered during the light cycle between 08:00 and 10:00.

## 2.4 Behaviour

### 2.4.1 Depression: Locomotor activity – OFT

Reduced locomotor activity is a symptom of MD (Łojko & Rybakowski, 2017). The method of Sherif and Oreland (1995) was used. Individual rats were placed into a square arena (100 x 100 x 50 cm), facing the centre of the arena. The test was conducted in a dimly lit room illuminated with red light (40 W). Animal behaviour was recorded for 5 minutes using a ceiling-mounted digital camera. The video files were analysed using Noldus Ethovision XT software (Noldus® Information Technology, Wageningen, The Netherlands), which calculated and reported the total distance (cm) travelled within the arena.

### 2.4.2 Depression: Despair – FST

We used a method as previously described (Schoeman *et al.*, 2017). Individual rats were placed in transparent, Perspex® swim tanks containing water at ambient temperature (25°C) and allowed to swim for 7 minutes. This was digitally recorded for behavioural analysis later. No pre-swim was applied as FSL rats already present with heightened immobility behaviour in the FST (Overstreet & Wegener, 2013). At the end of the 7-minute period, the rats were removed from the cages, dried and returned to their home cages. The first and last minute of the video files were excluded from the analysis, for reasons noted earlier (Oberholzer *et al.*, 2018). Immobility (despair), swimming (survival, coping) and climbing (escape-driven behaviour) behaviours were scored manually by a researcher blinded to treatment and expressed as time (seconds) spent performing each behaviour.

### 2.4.3 Social Interaction Test (SIT)

Social deficits are a prominent feature of MD (Łojko & Rybakowski, 2017). The social interaction test/task (SIT) was performed to assess anxiety-related social withdrawal behaviour in rodents (File & Seth, 2003; Kaidanovich-Beilin *et al.*, 2011). Behaviours are described in Table 1.

The SIT was conducted in the same arena and under the same lighting conditions as described in the OFT. Individual animals were allowed to revisit the arena the day prior to the SIT in order to habituate to their surroundings. The familiar arena and dim lighting are conducive to maximum

active behaviours (Wilson & Koenig, 2014). A pair of rats of similar mass (± 10 g) from the same treatment group and reared under the same conditions but unfamiliar to each other, were placed in the middle of the open field arena facing each other. Social behaviour was recorded using a digital camera mounted above the arena. These social behaviours are strongly correlated to that evident in human MD (Fone & Porkess, 2008). After each test session, any faecal boli were removed, urine wiped, and the arena cleaned with 10% ethanol solution. Behaviour in the arena was later manually scored from videos over a 10 min observation period by an observer blind to the treatment and rearing conditions. Since the behaviour of each rat is related to its partner in the arena, pair scores (thus n=6) were used (del Angel Ortiz *et al.*, 2016), with each behaviour expressed as the percentage (%) time of the total duration of the session.

**Table 1:** Depression-related social behaviour scored in the social interaction test (Adapted from Barnett (1958); Brain *et al.* (1989)).

| Category | Behaviour | Description |
|---|---|---|
| Social | Sniffing | Sniffing the head, snout, anogenital area, or body of the partner |
| | Approaching | Walking directly toward the partner |
| | Following | Moving in close proximity to the partner as it walks around the arena |
| | Grooming (allo-grooming) | Grooming the body of the partner using the mouth |
| | Crawling over/under | Both forepaws placed on the partner, with the head and anterior part of the body pushed underneath the partner |
| Asocial (anxiety) | Exploring | Walking or running around the arena, not obviously directed toward the partner, supported or unsupported rearing unrelated to partner |

## 2.5 Bioanalysis

### 2.5.1 Preparation of plasma and brain tissue

Rats were decapitated, and the frontal cortex and hippocampus were immediately dissected out on an ice-cooled glass slab as previously described (Mokoena *et al.*, 2015; Möller *et al.*, 2013). Trunk blood was collected in pre-chilled, 4 mL vacutainer tubes (Vacuette®) containing $K_3$EDTA solution as anti-coagulant. The blood was centrifuged at 1000 × g at 4°C for 15 min. Both the brain parts and plasma were fixed in liquid nitrogen and stored at −80°C until the day of analysis.

### 2.5.2 Monoamine quantification

NE, 5-HT, and DA were quantified in the hippocampus and frontal cortex using a high-performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (Viljoen *et al.*, 2018). Whole and regional brain monoamine analyses reflect their total extracellular and unreleased levels (Brand & Harvey, 2017b). An Agilent 1200 series HPLC (Agilent Technologies Inc., Santa Clara, CA USA), equipped with an isocratic pump and autosampler coupled to an ESA Coulochem III Electrochemical detector with a coulometric flow cell (Model 5011A High Analytical Cell and Guard cell 5020) and Chromeleon® Chromatography Management System version 6.8 (obtained from Thermo Fisher Scientific, Waltham, MA USA), was used for this analysis.

### 2.5.3 Plasma biochemistry: DBH, IL-6, TNF-α, and CORT

DBH (Catalogue no: abx256508, Abbexa, Cambridge, UK), CORT (Catalogue no: E-EL-R0269, Elabscience Biotechnology Inc., Wuhan China), IL-6 (Catalogue no: E-EL-R0015, Elabscience Biotechnology Inc., Wuhan China), and TNF-α (Catalogue no: E-EL-R0019, Elabscience Biotechnology Inc., Wuhan China) were measured by sandwich ELISA kits according to the manufacturer's protocol.

## 2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism® 8 for Windows (GraphPad Software Inc., San Diego, CA, USA) under the supervision of the Statistical Consultation Service of the NWU. All data were checked for normality using Shapiro-Wilk's test. Two-way ANOVA followed by

Bonferroni post-hoc test was applied in comparisons of body weight, behaviour, and biochemistry. The two-way ANOVA was selected to test the interaction between treatment and rearing condition as these were the only factors relevant to our hypothesis and aims of developing a TRD model and evaluating its response to FLX treatment. Significance was set at $p < .05$ for all comparisons. Where statistical data was narrowly missed i.e. $.05 \leq p \leq .06$ for a parameter that would highlight specific differences between SAL- or FLX-treated FSL and FSL-SIR animals, a t-test was performed. For all data, if no statistical significance was evident following the post-hoc test, a Cohen's *d* value was calculated to establish the effect size and practical significance. Large effect sizes are indicated by $d \geq 0.8$ and very large effect sizes by $d \geq 1.2$ (Sawilowsky, 2009). Only large and very large effect sizes are noted in the figures and discussed. Data are graphically presented as mean ± SEM.

## 3. Results

### 3.1 Body weight

Significant interaction between rearing conditions and treatment [$F(52, 715) = 6.769$, $p < .0001$] as well as significant main effects of treatment [$F(4.244, 233.4) = 749.7$, $p < .0001$] and rearing condition [$F(4, 55) = 21.51$, $p < .0001$] were observed. As illustrated in Figure 2A, SAL-treated and FLX-treated FSL and FSL-SIR rats were all significantly heavier than SD-SAL rats (all $p < .0001$). While SAL-treated FSL-SIR ($p < .0001$) and FLX-treated FSL-SIR ($p = .0001$) rats were significantly heavier than SAL-treated FSL rats, FLX-treatment caused decreased weight gain compared to SAL-treatment in FSL animals ($p < .0001$). FLX-treated FSL-SIR rats were significantly heavier than FLX-treated FSL ($p < .0001$) animals. Overall rate of weight gain in SIR-exposed, SAL-treated FSL lagged behind socially-reared, SAL-treated FSL rats (13.7% vs. 15.2%, Figure 2B), which a t-test found to be significant ($p<.0001$). When these groups were treated with FLX, FSL-SIR were observed to show more rapid weight gain than FSL-SAL (11.5% vs. 10.5%, Figure 2B), also found to be significant following a t-test ($p<.0001$).

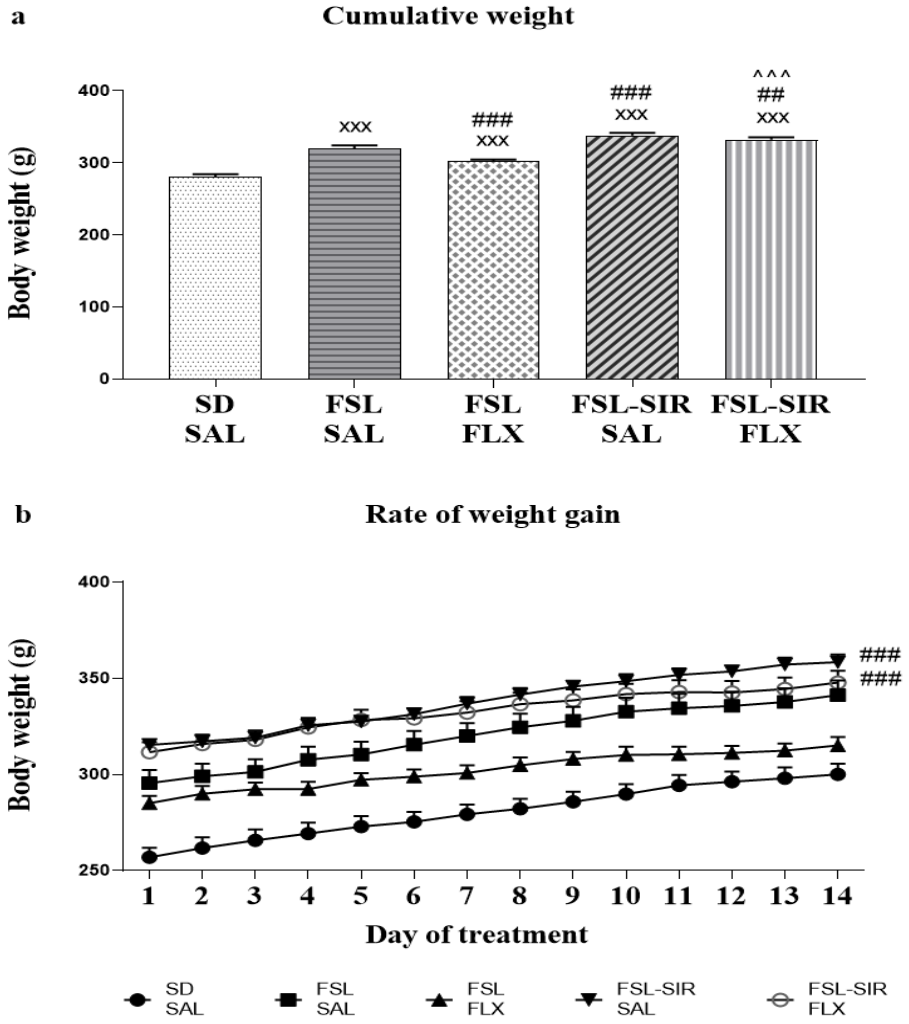



**Figure 2: (a)** Mean cumulative weight in SD, FSL, and FSL-SIR rats following treatment with SAL or FLX. $^{xxx}p$ < .0001 vs. SD-SAL; $^{###}p$ < .0001, $^{##}p$ < .01 vs. FSL-SAL; $^{\wedge\wedge\wedge}p$ < .0001 vs. FSL-FLX. **(b)** Rate of weight gain during the treatment period. $^{###}p$ < .0001 vs. FSL-SAL. Data are represented as the mean of 12 animals. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test. Data are presented as mean ± SEM. Precise *p* –values are presented in the text. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine.

### 3.2 OFT

Significant main effects of rearing condition [$F(4, 44) = 4.248$, $p = .0054$] and treatment [$F(11, 44) = 0.7036$, $p = .7283$], with no rearing condition x treatment interaction were revealed. As illustrated in Figure 3a, FLX-treated FSLs ($p = .0342$) and FLX-treated FSL-SIRs ($p = .0225$) travelled significantly less than SD-SAL rats. Cohen's *d* analysis showed a very large effect size decrease in distance

travelled by SAL-treated FSLs compared to SD-SAL rats ($d$ = 1.5). A large effect size decrease in locomotor activity was observed in FLX-treated FSL-SIR rats compared to SAL-treated FSL-SIRs ($d$ = 1.0), while a large effect size increase in activity was observed in SAL-treated FSL-SIR rats compared to FSL-SAL ($d$ = 0.9).

### 3.3 FST

*Immobility* (Figure 3b). A significant main effect of rearing condition [$F(4, 44) = 12.24$, $p < .0001$] but no rearing condition x treatment interaction or main effect of treatment was observed. Significantly increased immobility was observed in FSL-SAL ($p < .0001$), FSL-SIR-SAL ($p = .0078$), and FSL-SIR-FLX ($p < .0001$) compared to SD-SAL rats. FLX–treatment significantly decreased immobility in the FSL rats compared to FSL-SAL ($p = .0019$). FLX-treatment significantly raised immobility in FSL-SIR animals compared to FLX-treated FSL rats ($p = .0007$). Cohen's $d$ showed a large effect size decrease in immobility in FSL-SIR-SAL compared to FSL-SAL ($d$ = 1.1).

*Swimming* (Figure 3c). A significant main effect of rearing condition [$F(4, 44) = 13.34$, $p < .0001$] was indicated although there was no rearing condition x treatment interaction or main effect of treatment. SAL-treated FSLs ($p < .0001$) and FLX-treated FSL-SIR rats ($p = .0229$) spent significantly less time swimming compared to SD-SAL rats. A significant increase in swimming was observed in FSL-SIR-SAL rats ($p = .0064$), FSL-FLX ($p < .0001$) and FSL-SIR-FLX ($p = .0262$) rats compared to SAL-treated FSLs. Cohen's $d$ showed a very large effect size decrease in swimming in FLX-treated FSL-SIR compared to FLX-treated FSL ($d$ = 1.5).

*Climbing* (Figure 3d). A significant main effect of rearing condition [$F(4, 44) = 5.269$, $p = .0015$] was noted although there was no rearing condition x treatment interaction or main effect of treatment. FLX treatment significantly increased climbing in FSL-SIRs compared to SD-SAL rats ($p = .0005$). Cohen's $d$ showed a large effect size increase in climbing in SAL-treated FSL-SIR compared to SD-SAL ($d$ = 1.2). Similarly, a large effect size increase in climbing in FLX-treated FSL-SIR compared to FSL-FLX ($d$ = 1.0) and FSL-SAL ($d$ = 1.0).

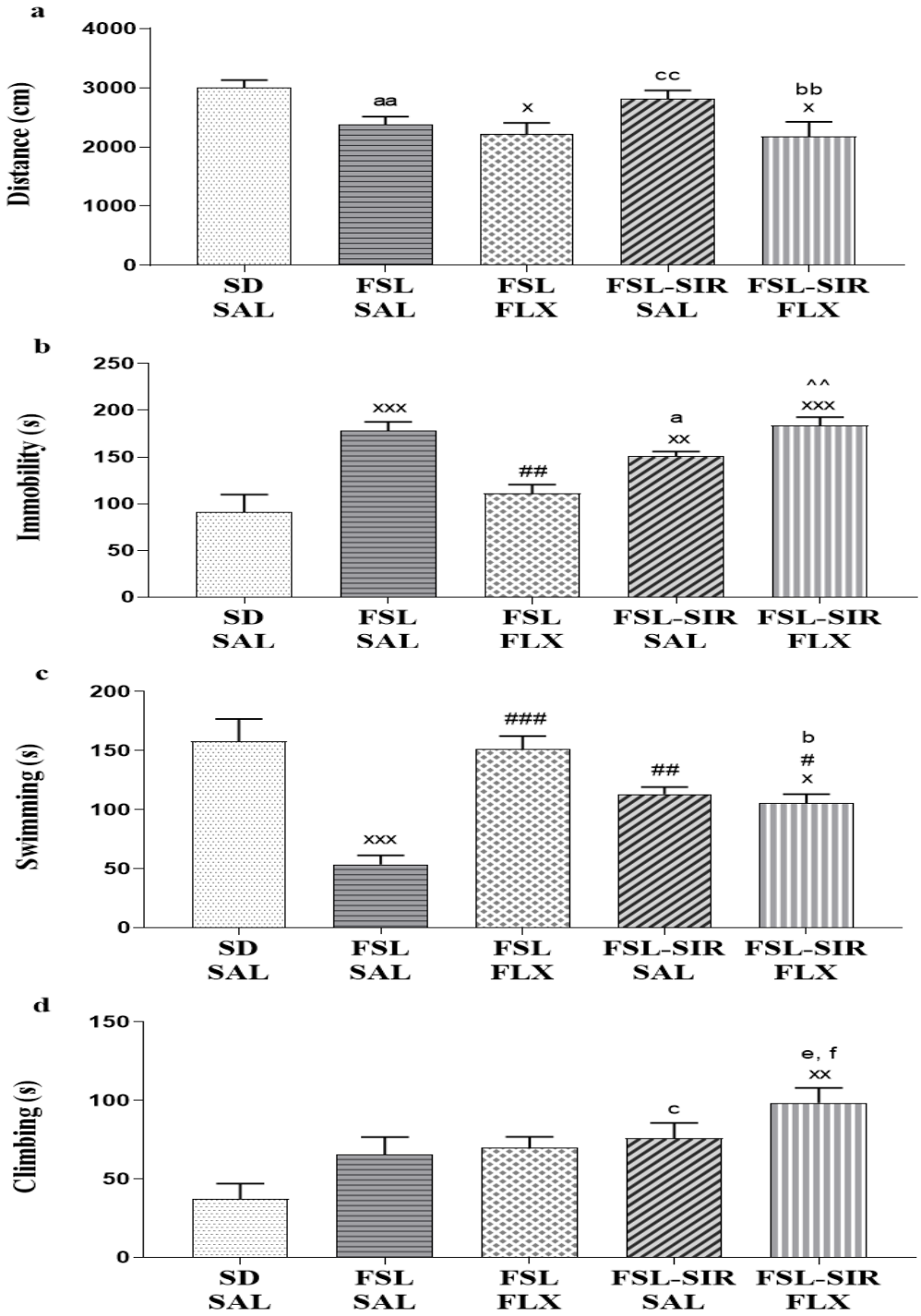



**Figure 3a:** Locomotor activity measured as distance travelled in the OFT. $^{x}p < .05$ vs. SD-SAL; $^{aa}d = 1.5$ vs. SD-SAL, $^{cc}d = 0.9$ vs. FSL-SAL; $^{bb}d = 1.0$ vs. FSL-SIR-SAL. **Figure 3b-d:** Immobility and swimming and climbing behaviours as measured in the FST. **(b)** Immobility (s). $^{xxx}p < .0001$, $^{xx}p < .01$ vs. SD-SAL; $^{\#\#}p < .01$ vs. FSL-SAL; $^{\wedge\wedge}p < .01$ vs. FSL-FLX; $^{a}d = 1.1$ vs. FSL-SAL. **(c)** Swimming (s). $^{xxx}p < .0001$, $^{x}p < .05$ vs. SD-SAL; $^{\#\#\#}p < .0001$, $^{\#\#}p < .01$, $^{\#}p < .05$ vs. FSL-SAL; $^{b}d = 1.5$ vs. FSL-FLX. **(d)** Climbing (s). $^{xx}p < .01$ vs. SD-SAL; $^{c}d = 1.2$ vs. SD-SAL, $^{f}d = 1.0$ vs. FSL-SAL, $^{e}d = 1.0$ vs. FSL-FLX. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. Detailed *p* –values in the text. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine.

### 3.4 SIT

*Social* (amicable; Figure 4a). A significant main effect of treatment [$F(29, 116) = 17.85$, $p < .0001$] and rearing condition [$F(4, 116) = 7.317$, $p < .0001$] was revealed although there was no treatment x rearing condition interaction. A significant decrease in social behaviour was observed in FSL-SAL ($p < .0001$) and FSL-SIR-SAL ($p = .0304$) rats compared to SD-SAL rats. FLX treatment significantly increased social behaviour in FSL-SIR compared to SAL-treated FSL animals ($p = .0007$).

*Asocial* (social anxiety; Figure 4b). A significant main effect of rearing condition [$F(4, 20) = 10.07$, $p = .0001$] was observed without significant treatment x rearing condition interaction and no main effect of treatment was noted. A significant increase in asocial behaviour was observed in FSL-SAL ($p = .0139$), FSL-SIR-SAL ($p = .0003$), and FSL-SIR-FLX ($p = .0005$) groups compared to SD-SAL animals. FSL-SIR-SAL rats exhibited significantly more asocial behaviour compared to FSL-FLX ($p = .0388$). FSL-SIR-FLX narrowly missed significance in a two-way ANOVA compared to FSL-FLX ($p = .0542$). To validate the treatment resistant nature of FSL-SIR rats to FLX, a t-test revealed a significant increase in asocial behaviour in FSL-SIR-FLX compared to FSL-FLX ($p = .0066$). Cohen's *d* analysis revealed a large effect size increase in asocial behaviour in FSL-SIR-SAL compared to FSL-SAL ($d = 1.1$). A very large effect size reduction of asocial behaviour was noted in FSLs following FLX treatment ($d = 1.7$).

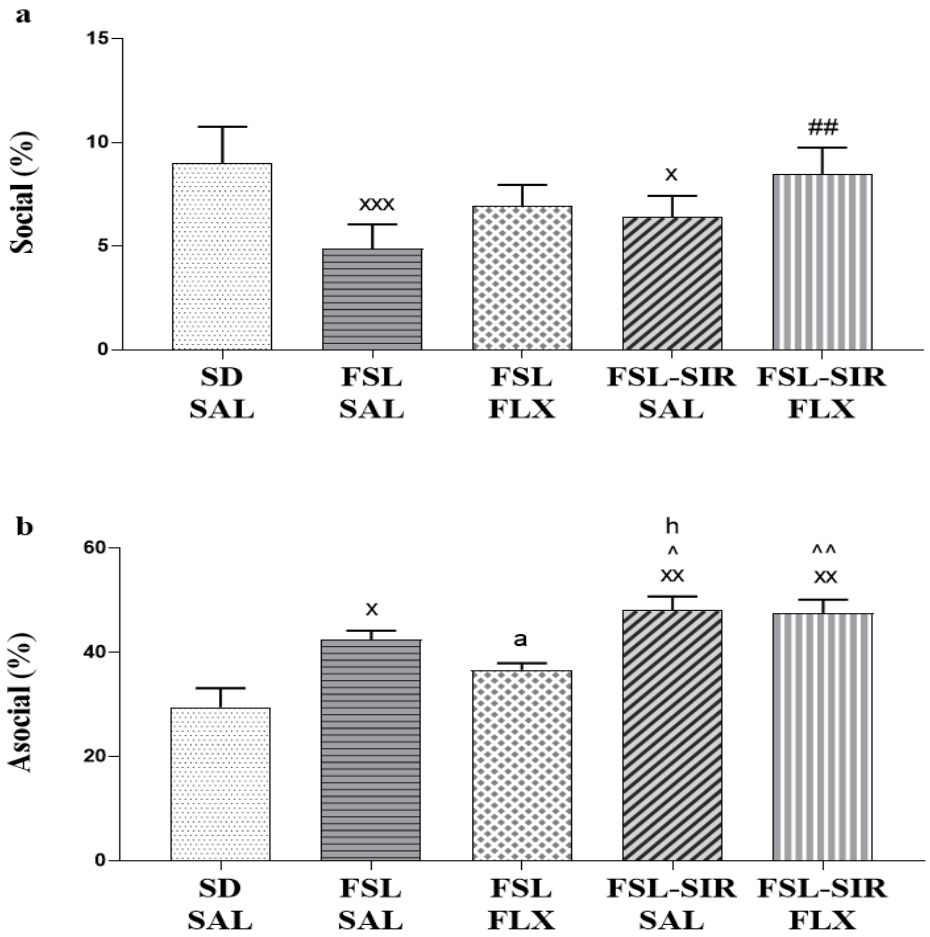



**Figure 4:** Social interactive behaviour as measured in the SIT. **(a)** Social (amicable) behaviour. $^{xxx}p < .0001$, $^{x}p < .05$ vs. SD-SAL; $^{\#\#}p < .01$ vs. FSL-SAL. **(b)** Asocial/socially anxious-like behaviour. $^{xx}p < .01$, $^{x}p < .05$ vs. SD-SAL; $^{\wedge\wedge}p < .01$, $^{\wedge}p < .05$ vs. FSL-FLX; $^{a}d = 1.7$, $^{h}d = 1.1$ vs. FSL-SAL. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis and Student's t-test with Welch's correction. Data are presented as mean ± SEM. Detailed *p* –values in the text. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine.

### 3.5 Monoamines

*NE: Frontal cortex* (Figure 5a). A significant main effect of rearing condition [$F(4, 44) = 84.57$, $p < .0001$] but with no main effect of treatment and no treatment x rearing condition interaction were revealed. Significantly reduced levels of frontocortical NE were observed in FSL-SAL, FSL-FLX, FSL-SIR-SAL, and FSL-SIR-FLX animals compared to SD-SAL (all $p < .0001$). FSL-SIR-SAL animals were found to have significantly diminished NE levels in this brain region compared to FSL-SAL animals ($p < .0001$) and FSL-FLX rats ($p < .0001$). A significant increase in frontocortical NE was observed in the FLX-treated FSL-SIR rats compared to SAL-treated FSL-SIR ($p < .0001$).

*Hippocampus* (Figure 5b). A significant main effect of rearing condition [F(4, 44) = 20.08, p < .0001] was observed although no treatment x rearing condition interaction or main effect of treatment was indicated. Significantly reduced hippocampal NE levels were observed in FSL-SAL ($p$ = .0002), FSL-FLX ($p$ < .0001), FSL-SIR-SAL ($p$ < .0001), and FSL-SIR-FLX ($p$ = .0046) groups compared to SD-SAL rats. There was significantly diminished hippocampal NE levels in SAL-treated FSL-SIR compared to FSL-SAL ($p$ = .0018) and FSL-FLX ($p$ = .0036) rats. FLX treatment significantly increased hippocampal NE in FSL-SIR compared to SAL-treatment ($p$ < .0001).

*5-HT: Frontal cortex* (Figure 5c). A significant main effect of rearing condition [$F$(4, 44) = 21.63, $p$ < .0001] was noted although there was no treatment x rearing condition interaction or main effect of treatment. SAL-treated FSL-SIR rats presented with significantly reduced frontocortical 5-HT compared to SD-SAL rats ($p$ = .0002), FSL-SAL ($p$ = .0013), and FSL-FLX ($p$ < .0001). FLX-treated FSL-SIR animals had significantly elevated 5-HT levels in this region compared to SAL-treated FSL-SIR ($p$ = .0007) rats.

*Hippocampus* (Figure 5d). A significant main effect of rearing condition [$F$(4, 44) = 21.63, $p$ < .0001] was noted although there was no treatment x rearing condition interaction or main effect of treatment. SAL-treated FSL ($p$ = .0007), FSL-FLX and FSL-SIR-SAL rats (both $p$ < .0001) presented with significantly reduced 5-HT compared to SD-SAL rats. Significantly diminished hippocampal 5-HT was observed in FLX-treated FSL ($p$ = .0311) and SAL-treated FSL-SIR ($p$ = .0152) rats compared to SAL-treated FSL rats. SIR significantly raised 5-HT in this region of FSL-SIR brains compared to FLX-treated FSL ($p$ = .0003) and SAL-treated FSL-SIR ($p$ = .0001) rats. Cohen's $d$ analysis showed a large effect size reduction in hippocampal 5-HT in FLX-treated FSL-SIR rats compared to SD-SAL controls ($d$ = 1.0).

*DA: Frontal cortex* (Figure 5e). No significant differences between cohorts were found. Cohen's $d$ analysis showed a large effect size reduction in frontocortical DA in SAL-treated FSL-SIR compared to SD-SAL ($d$ = 0.8) and SAL-treated FSL rats ($d$ = 1.1), and a very large effect size reduction compared to FLX-treated FSL rats ($d$ = 3.5). FLX treatment caused a very large effect size increase in frontocortical DA in FSL-SIR compared to FSL-FLX rats ($d$ = 2.0) and a very large elevation compared to SAL-treated FSL-SIR rats ($d$ = 2.4).

*Hippocampus* (Figure 5f). Significant effects of treatment [$F$(11, 44) = 2.576, $p$ = .0129] and rearing condition [$F$(4, 44) = 2.784, $p$ = .0380] but without interaction between the two factors were

indicated. Hippocampal DA was significantly reduced in SAL-treated FSL-SIR rats compared to FLX-treated FSL rats ($p$ = .0490). Cohen's $d$ analysis -showed a very large effect size reduction in DA in SAL-treated FSL-SIR brains compared to SD-SAL rats ($d$ = 1.3). A large effect size reduction was also observed in FLX-treated FSL-SIR rats compared to FLX-treated FSL rats ($d$ = 1.5). FLX caused a large effect size increase in FSL-SIR rats compared to SAL-treated FSL-SIR rats ($d$ = 1.1).

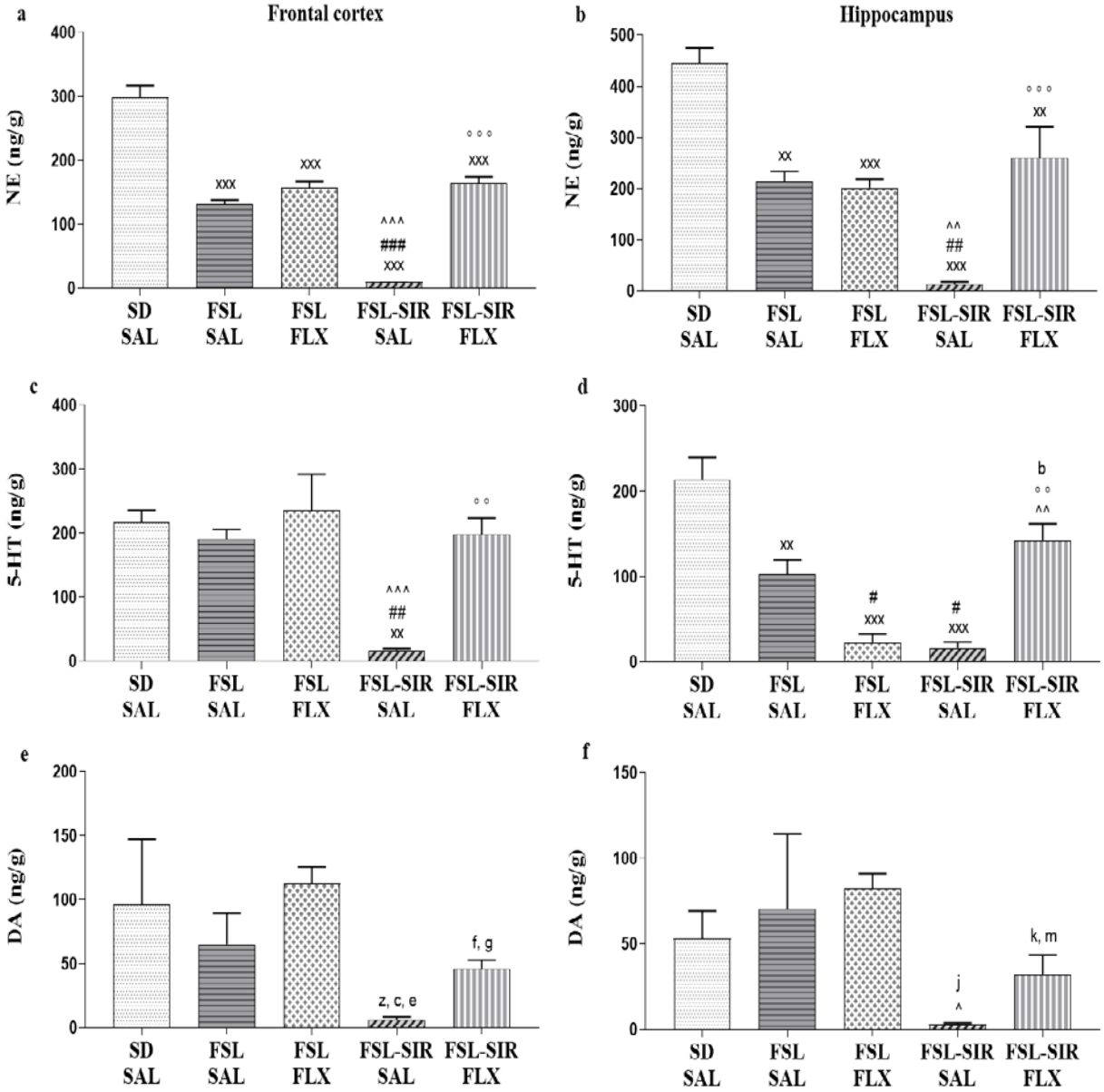



**Figure 5:** Monoamine levels in the frontal cortex (a, c, e) and hippocampus (b, d, f). **(a)** NE. $^{xxx}p$ < .0001 vs. SD-SAL; $^{###}p$ < .0001 vs. FSL-SAL; $^{\wedge\wedge\wedge}p$ < .0001 vs. FSL-FLX; $^{\circ\circ\circ}p$ < .0001 vs. FSL-SIR-SAL. **(b)** NE. $^{xxx}p$ < .0001, $^{xx}p$ < .01 vs. SD-SAL; $^{\#\#}p$ < .01 vs. FSL-SAL; $^{\wedge\wedge}p$ < .01 vs. FSL-FLX; $^{\circ\circ\circ}p$ < .0001 vs. FSL-SIR-SAL. **(c)** 5-HT. $^{xx}p$ < .01 vs. SD-SAL; $^{\#\#}p$ < .01 vs. FSL-SAL; $^{\wedge\wedge\wedge}p$ < .0001 vs. FSL-FLX; $^{\circ\circ}p$ < .01 vs. FSL-SIR-SAL. **(d)** 5-HT. $^{xxx}p$ < .0001, $^{xx}p$ < .01 vs. SD-SAL; $^{\#}p$ < .05 vs. FSL-SAL; $^{\wedge\wedge}p$ < .01 vs. FSL-FLX, $^{\circ\circ}p$ < .01 vs. FSL-SIR-SAL. $^{b}d$ = 1.0 vs. SD-SAL. **(e)** DA. $^{z}d$ = 0.8 vs. SD-SAL; $^{c}d$ = 1.0 vs. FSL-SAL, $^{e}d$ = 3.5, $^{f}d$ = 2.0 vs. FSL-FLX, $^{g}d$ = 2.4 vs. FSL-SIR-

SAL. **(f)** DA. $^{\wedge}p < .05$ vs. FSL-FLX. $^{j}d = 1.3$ vs. SD-SAL, $^{k}d = 1.5$ vs. FSL-FLX; $^{m}d = 1.1$ vs. FSL-SIR-SAL. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. Detailed *p* –values in the text. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine.

### 3.6 Plasma biochemistry

*DBH* (Figure 6a). A significant main effect of rearing condition [$F(4, 36) = 4.841$, $p = .0032$] was noted with no main effect of treatment and no interaction between these two factors indicated. SAL-treated FSL-SIR ($p = .0350$) and FLX-treated FSL-SIR ($p = .0166$) animals presented with significantly reduced levels of plasma DBH compared to SD-SAL rats. Cohen's *d* showed very large effect size reductions in plasma DBH in SAL-treated FSL-SIR compared to SAL-treated FSL animals ($d = 1.9$) and FSL-FLX animals ($d = 1.8$) and in FLX-treated FSL-SIR compared to SAL-treated FSL ($d = 2.1$) and FLX-treated FSL animals ($d = 2.0$).

*CORT* (Figure 6b). A significant main effect of rearing condition [$F(4, 36) = 3.412$, $p = .0183$] was indicated but with no main effect of treatment or treatment x rearing condition interaction. FLX-treated FSLs presented with significantly elevated plasma CORT levels compared to SD-SAL ($p = .0276$) and SAL-treated FSLs ($p = .0335$). SIR of FSL rats increased CORT levels versus SD-SAL and FSL-SAL rats by a large effect size (both $d = 0.8$). A large effect size reduction of plasma CORT in FLX-treated FSL-SIR animals compared to FLX-treated FSL animals ($d = 0.8$), as revealed by Cohen's *d* analysis.

*IL-6* (Figure 6c). No significant differences between cohorts were found.

*TNF-α* (Figure 6d). No significant differences between cohorts were revealed. Cohen's *d* showed a large effect size reduction of plasma TNF-α in FSL-SIR-FLX rats compared to SD-SAL rats ($d = 1.0$), SAL-treated FSL rats ($d = 0.8$), and FLX-treated FSL animals ($d = 0.9$).

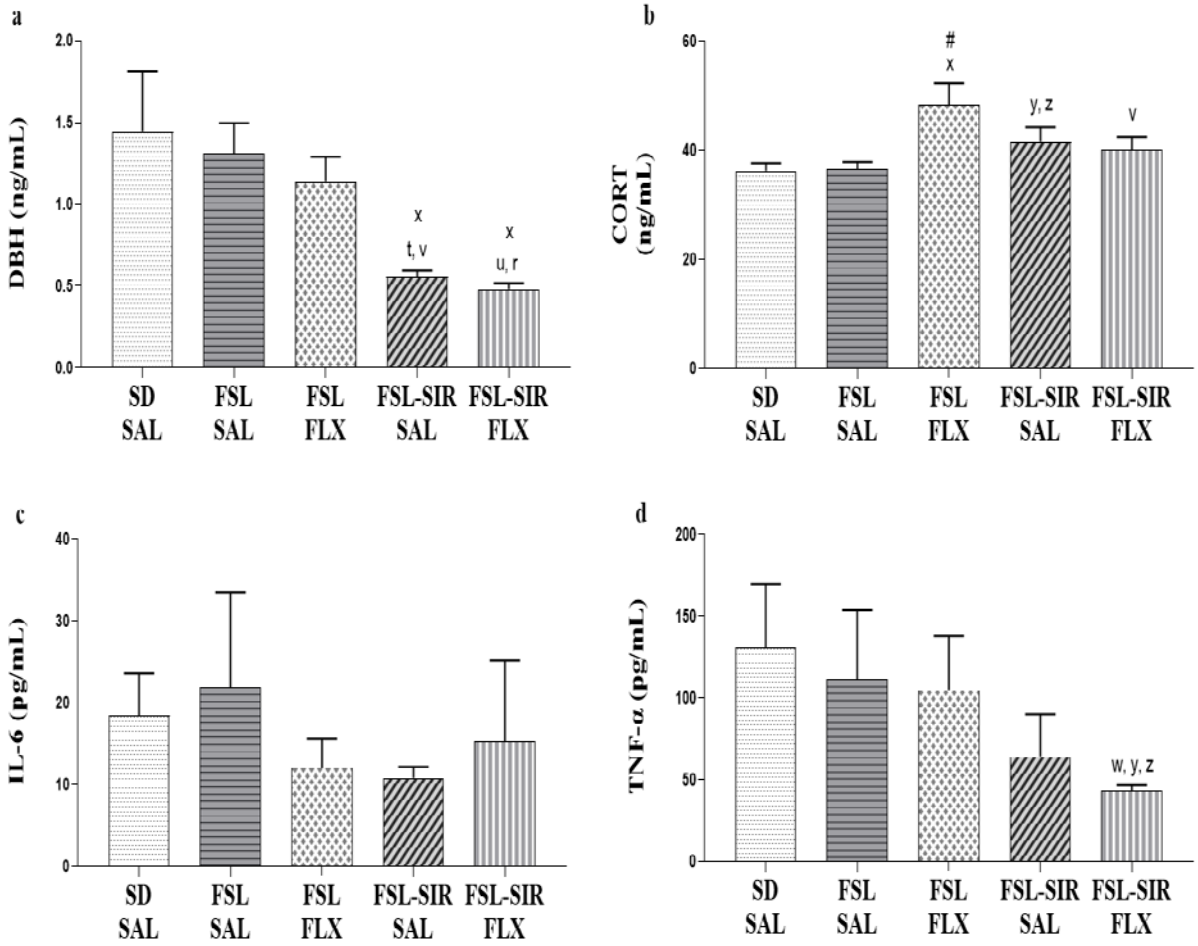



**Figure 6:** Plasma biochemistry. **(a)** DBH. $^{x}p < .05$ vs. SD-SAL; $^{t}d = 1.9$, $^{r}d = 2.1$ vs. FSL-SAL; $^{v}d = 1.8$, $^{u}d = 2.0$ vs. FSL-FLX. **(b)** CORT. $^{x}p < .05$ vs. SD-SAL; $^{\#}p < .05$ vs. FSL-SAL; $^{y}d = 0.8$ vs. SD-SAL; $^{z}d = 0.8$ vs. FSL-SAL; $^{v}d = 0.8$ vs. FSL-FLX. **(d)** TNF-α. $^{y}d = 1.1$ vs. SD-SAL; $^{w}d = 0.8$ vs. FSL-SAL; $^{z}d = 0.9$ vs. FSL-FLX. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. Detailed *p*–values in the text. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine.

## 4. Discussion

Key findings are that FSL rats exposed to SIR presented with depressive-like symptoms manifesting as reduced swimming and prolonged immobility in the FST similar to socially-reared FSL rats. However, these behaviours *worsened* in response to FLX, while worse social impairment in FSL-SIR rats also showed non-response to FLX. Biochemically, FSL-SIR rats presented with diminished DA, NE and 5-HT in both the frontal cortex and hippocampus, which were raised by FLX, as well as had

reduced plasma DBH which was unaffected by FLX. Together these data indicate treatment resistance to a first-line antidepressant.

Reduced body weight of FSL rats is well-described (Overstreet, 1993), with FSL and FSL-SIR rats gaining weight more slowly than SD-SAL rats (data not shown), congruent with similar animal models (Willner, 2017). Sustained weight loss is typical of chronic stress, such as SIR (Kirkedal *et al.*, 2019), as evident in SAL-treated FSL-SIR rats. Interestingly, patients with TRD have a higher BMI and presence of obesity (Liao *et al.*, 2019; Rizvi *et al.*, 2014). FLX treatment reduced weight gain in social and isolated FSL rats, in line with its anorexigenic effects (Silva *et al.*, 1999), although like in patients with TRD (Rizvi *et al.*, 2014), FLX-treated FSL-SIR rats gained weight significantly faster than SAL-treated FSL rats.

Consistent with literature (Overstreet *et al.*, 2005), FSL rats displayed psychomotor retardation in the OFT compared to SD-SAL rats ($d$ = 1.5, Figure 3a), with no significant locomotor effects evident in FSL-SIR versus SD-SAL rats (Figure 3a). However, consistent with TRD versus MD patients (Malhi *et al.*, 2019; Schatzberg & Rothschild, 1992), locomotor activity was increased in SAL-treated FSL-SIR rats versus FSL-SAL rats (Figure 3a), which in turn was significantly decreased by FLX in FSL and FSL-SIR animals (Figure 3a).

FSL rats displayed significantly elevated immobility (despair) and reduced swimming (survival behaviour) in the FST versus SD-SAL rats as well as being reversed by FLX (Figure 3b, c), indicative of a 5-HT-selective antidepressant. FSL-SIR rats also displayed marked immobility and reduced swimming versus SD-SAL animals, although immobility was less than in FSL-SAL animals ($d$ = 1.1). However, the antidepressant effects of FLX were negated in FSL-SIR rats, with significantly increased immobility and decreased swimming versus SD-SAL rats (Figure 3b, c). Climbing behaviour was largely unaffected by condition or treatment in FSL-SAL rats (Figure 3d), but was raised by SIR and FLX in FSL-SIR which argues *against* increased immobility being a confounding variable in the FST in FLX-treated FSL-SIR rats.

Consistent with literature (Liebenberg *et al.*, 2012; Overstreet *et al.*, 2005), FSL rats displayed social withdrawal (Figure 4a), a key symptom of MD (American Psychiatric Association 2013), and increased arena exploration in the SIT (Figure 4b), the latter a measure of anxiety (del Angel Ortiz

*et al.*, 2016; Elliott & Grunberg, 2005). Anxiety and depression are notoriously comorbid (Samuels *et al.*, 2011), and although not a core trait of the FSL rat (Overstreet & Wegener, 2013), anxiety has been noted in the SIT (Overstreet *et al.*, 2005) as re-affirmed here (Figure 4b). Moreover, and congruent with literature (Dulawa *et al.*, 2004; Farhan & Haleem, 2016), these anxiety-like behaviours were effectively reversed by FLX ($d$ = 1.7, Figure 4b). Social deprivation decreases social interactive behaviour and increases anxiety in adulthood (Regenass *et al.*, 2018). Not surprising then that FSL-SIR animals exhibited greater social anxiety-like behaviour than the FSL group ($d$ = 1.1). Similarly, FSL-SIR rats exhibited disordered social behaviour and asocial (anxious) behaviour versus SD-SAL rats (Figures 4a, b). Although SSRIs are anxiolytic in FSL rats (Dulawa *et al.*, 2004; Farhan & Haleem, 2016), see also Figure 4b, FLX showed no anxiolytic effect in FSL-SIR rats (Figure 4b), which alludes to an underlying neurobiological change following SIR that prompts resistant to the anxiolytic effects of chronic FLX treatment.

The monoamine hypothesis suggests that depressive symptoms result from deficits in NE and 5-HT neurotransmission (Smolders *et al.*, 2008; Villas Boas *et al.*, 2019), a resulting upregulation of 5-HT receptors (5-$HT_{1A, 2A, 2C, 4}$) (Ahrold & Meston, 2009; Amidfar *et al.*, 2018; Yohn *et al.*, 2017), and up-regulation and increased sensitivity of NE receptors (particularly $\alpha_2$ adrenergic) (Cottingham & Wang, 2012; Villas Boas *et al.*, 2019). FSL rats present with a blunted 5-HT response (Overstreet *et al.*, 2005), reduced 5-HT transporters (SERT) (Kovacevic *et al.*, 2010) as well as elevated cortico-hippocampal 5-HT (Overstreet & Wegener, 2013; Zangen *et al.*, 1997). Here, frontocortical 5-HT in the FSL rats was unperturbed. FSL rats displayed reduced 5-HT in the hippocampus (Figure 5d), in agreement with the biogenic amine theory of MD. On the other hand, juvenile adversity (e.g. SIR) reduces the density and attenuates function of post-synaptic 5-$HT_{1A}$ receptors in the hippocampus and stress centres of the brain (Kuramochi & Nakamura, 2009; Matsuzaki *et al.*, 2011; Muchimapura *et al.*, 2003) and reduces presynaptic serotonergic function (Muchimapura *et al.*, 2003). This would explain the significantly reduced cortico-hippocampal 5-HT levels in FSL-SIR versus SAL-treated FSL rats (Figure 5c, d). That said, although depletion of 5-HT has been shown to block the action of SSRIs (Willner *et al.*, 2013), FSL-SIR rats treated with FLX still presented with significantly increased cortico-hippocampal 5-HT (Figure 5c, d) yet now displayed *exacerbated* depressive behaviours (Figure 3b). This could speak to the bidirectional role of $5HT_{1A}$ receptors in the response to stress (Harvey *et al.*, 2004), where elevated 5-HT has been suggested to have opposing actions on mood (Andrews *et al.*, 2015).

FSL rats also presented with reduced NE in the frontal cortex and hippocampus versus SD-SAL controls (Figure 5a, b) correlating with the monoaminergic theory of MD. However, FLX did not alter this, nor was there an effect on climbing behaviour (Figure 3d), thus excluding a possible noradrenergic mode of action (Cryan & Slattery, 2007). Similarly, FSL-SIR-SAL animals presented with reduced cortico-hippocampal NE versus both SD-SAL and SAL-treated FSL groups, although levels were further and significantly reduced in FSL-SIR rats versus FSL-FLX rats (Figure 5a, b). Hyponoradrenergia is associated with social withdrawal (Swanepoel *et al.*, 2018), supportive of the social impairment seen in FSL and FSL-SIR rats (Figure 4a, b). Psychosocial impairments are also highly prevalent in TRD (Petersen *et al.*, 2004) and correlate with low cortico-hippocampal NE levels in FSL-SIR rats. FLX aided in the recovery of NE in FSL-SIR rats, supportive of the increased climbing of FSL-SIR animals in the FST (Figure 3d), although with only slight improvement in social behaviour in FSL-SIR rats versus FSL-SAL rats.

FSL rats presented with unaltered cortico-hippocampal DA levels versus SD-SAL controls (Figure 5e, f), corresponding with previous findings (Tillmann *et al.*, 2018). However frontocortical ($d$ = 0.8) and hippocampal ($d$ = 1.3) DA levels were reduced in FSL-SIR versus SD-SAL rats (Figure 5e, f), congruent with the monoaminergic theory of depression (Villas Boas *et al.*, 2019), as well as being evidence in patients with TRD (Hori & Kunugi, 2012; Wijeratne & Sachdev, 2008). FLX increases cortical and hippocampal DA (Kobayashi *et al.*, 2012; Zhang *et al.*, 2000), also evident in FSL-SIR versus FSL-SIR-SAL rats (Figure 5e, f). These data are intriguing and warrant further study using a model that co-presents with depression and psychosis-like behaviour.

As in MD literature (Hess *et al.*, 2009), plasma DBH levels were unchanged in FSL versus SD-SAL rats (Figure 6a), also unaffected by FLX treatment. However, DBH was significantly reduced in FSL-SIR-SAL versus SD-SAL rats, with a very large effect size reduction versus SAL-treated FSL rats, not unlike that described in the clinical literature (Meltzer *et al.*, 1976; Meyers *et al.*, 1999). With reduced DBH being a putative biomarker of TRD (Willner & Belzung, 2015), these findings confirm our earlier monoamine data, especially DA, as well as behavioural data supporting a TRD animal model. While FLX treatment elevated cortico-hippocampal DA levels in FSL rats (Figure 5e, f), it did not change DBH (Figure 6a), as noted elsewhere (Meyers *et al.*, 1999). Elevated DA could therefore be a direct consequence of insufficient DBH conversion from NE. The inability of FLX to reverse

lowered DBH levels in FSL-SIR animals suggests a SIR-induced change to FSL neurobiology that resists response to FLX.

FSL rats presented with unaltered basal plasma CORT levels versus SD-SAL rats (Figure 6b), not unusual in this model (Serova *et al.*, 1998). TRD is associated with elevated CORT (Caraci *et al.*, 2018), supported by findings in the FSL-SIR model (Figure 6b). Interestingly, FLX treatment exacerbated the CORT response in FSL rats (Figure 6b). FLX treatment of ≤2 weeks may have a stimulatory effect on the rodent HPA-axis (Pariante *et al.*, 2004), possibly by up-regulating glucocorticoid receptors (Heydendael & Jacobson, 2010). Indeed, sub-chronic (9 days) FLX treatment has been found to down-regulate glucocorticoid receptor expression and to increase CORT (Pariante *et al.*, 2004). Unlike in FSL-SAL rats, plasma CORT was unaffected by FLX treatment (Figure 6b).

Elevated IL-6 and TNF-α have been causally linked to MD (Villas Boas *et al.*, 2019), while elevated IL-1α has been described in FSL rats (Carboni *et al.*, 2010). Here, neither plasma IL-6 nor TNF-α were altered in FSL versus SD-SAL rats, and were unaffected by FLX (Figure 6c, d). Although elevations in TNF-α and IL-6 are described in MD, their role in predicting antidepressant resistance is debated (Perlman *et al.*, 2019). However, while FLX had no effect on IL-6 in FSL or FSL-SIR rats, it engendered a large effect size decrease in TNF-α in FSL-SIR versus SD-SAL and FLX-treated FSL rats (Figure 6d). FLX has anti-inflammatory effects via serotonergic transmission and activation of the HPA-axis (Bianchi *et al.*, 1994; Roumestan *et al.*, 2007), which we also observed in FSL rats (Figure 6b). That FSL-SIR rats did not exhibit significant elevations in these cytokines or plasma CORT may point towards inherent biological mechanisms that protect against severe combined insults. This is especially evident in dual-hit models, e.g. (Goh *et al.*, 2020). In this regard, an adverse early-life experience is said to trigger an adaptive process that renders an individual better adapted or resilient to stressful environments later in life (Daskalakis *et al.*, 2012; Seery *et al.*, 2013; Vargas *et al.*, 2016).

In summary, FSL-SIR rats were significantly heavier than FSL rats in response to treatment, a symptom of TRD (Rizvi *et al.*, 2014), and where weight loss is typically seen following FLX-treatment, this was not demonstrated in FSL-SIR rats. FSL-SIR rats also showed a large effect size increase in locomotor activity versus FSL rats, consistent with TRD literature (Keller *et al.*, 2007; Schatzberg &

Rothschild, 1992). TRD presents with exaggerated symptoms of MD in addition to antidepressant resistance (Keller *et al.*, 2007; Schatzberg *et al.*, 2014). FSL-SIR rats presented with reduced coping behaviours, increased depressive-like behaviour, and increased social withdrawal and social anxiety, together with altered cortico-hippocampal NE and 5-HT levels supportive of deficits in coping and escape-directed strategies. Although FSL-SIR rats showed similar behaviour deficits to FSL rats, they were not more severe. Importantly, while biogenic amine anomalies were abrogated by FLX, social deficits remained unresponsive while depressive symptoms worsened following FLX treatment. In addition, FSL-SIR rats demonstrated a large effect size increase in CORT and a very large effect size reduction in DBH levels, recognised biomarkers of TRD (Caldarone *et al.*, 2015; Willner & Belzung, 2015), which FLX also failed to reverse. However, FSL-SIR rats did not exhibit significantly raised plasma inflammatory cytokine levels, although FLX tended to lower plasma TNF-alpha levels.

**Limitations of the study**

SIR is known to produce psychotic-like symptoms while some forms of TRD present with co-occurring psychotic symptoms. It is possible that underlying psychotic-like manifestations are present in FSL-SIR rats. Indeed, reduced fronto-cortical DA and reduced plasma DBH allude to this possibility. Such behaviours would not be evident within the range of behavioural tests performed in this paper and should be considered in future work. In addition, response to an atypical antipsychotic with/without an SSRI is recommended to improve predictive validity.

In conclusion, exposure of a genetic animal model of MD to post-weaning SIR results in a more intractable depressive-like phenotype as well as changes in TRD-related biomarkers, that are resistant to traditional antidepressant treatment.

## 5. Declaration of Competing Interest

With respect to this work, the authors declare that over the past three years, BHH has participated in advisory boards and received honoraria from Servier and Lundbeck, and has received research funding from Servier, Lundbeck, and HG&H Pharma. The authors declare that, except for income from the primary employer and research funding to BHH from the above-mentioned organizations and agencies, no financial support or compensation has been received from any individual or

corporate entity over the past three years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest. KM and MW have no conflicts of interest to declare.

## 6. Contributors

KM designed the study treated the animals and collected the samples, performed behavioural and bioanalytical procedures as well as the statistical analysis, interpreted the results and prepared the first draft as well as the final version of the manuscript. MM advised on the design of the study and on setting up of the social isolation rearing and assisted with the statistical analysis. BHH devised the concept of the study, advised on the design of the study, supervised KM, interpreted the results, co-wrote the manuscript and prepared it for submission. All the authors read and approved the final version of the manuscript for submission.

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# CHAPTER 4: MANUSCRIPT B

**Preamble**

This chapter presents the full-length manuscript titled: **Bio-behavioural validation of a novel neurodevelopmental animal model of treatment resistant depression: Response to olanzapine with/without fluoxetine** and prepared for submission to *Frontiers in Psychiatry, Animal Models of Psychiatry* section published by Frontiers Media SA. Instructions to the author regarding online submission and formatting requirements of manuscripts are available at: https://www.frontiersin.org/about/author-guidelines. For the sake of ease of readability, the format and layout of this chapter is in accordance with the above journal's requirements, albeit slightly amended to comply with the rest of the thesis. Thus, the figure legends and tables are inserted into the text with their corresponding paragraphs rather than after the references as stipulated in the author guidelines of this journal.

According to research Question 2 (see Chapter 1 section 3.2), and following initial face, construct, and predictive validation the FSL-SIR rat as a putative model of TRD (Manuscript A, Chapter 3), this manuscript reports on the further validation of this model, but now specifically with regard to possible congruence with a tentative animal model of MDpsy (see *Figure 1: Study design* in this Manuscript). This was done by exposing the FSL rat to post-weaning social isolation rearing (SIR), a neurodevelopmental paradigm of psychosis/SCZ, with the aim of inducing psychotic-like behaviour in an animal shown to exhibit depressive-like behaviour and poor response to FLX treatment (Manuscript A). This manuscript reports MDpsy-related bio-behavioural changes in the FSL-SIR model of MDpsy and evaluates the model's response to the antipsychotic, OLZ, and to OLZ+FLX combination treatment, or OFC. The FSL-SIR model is compared to SAL-treated SD rats to establish baseline bio-behavioural anomalies, and is then compared to SAL-treated, SIR-SD rats to show similarities to and differences from a typical validated model of SCZ/psychosis. OLZ-treated FSL-SIR rats are compared to SAL-treated SD and SD-SIR rats as well as OLZ-treated SD-SIR rats to determine the extent of reversal or resistance of the presenting bio-behavioural parameters to standard antipsychotic (OLZ) treatment and combined antipsychotic-antidepressant treatment (i.e. OLZ+FLX, or OFC) relative to these controls, the latter having been shown useful in treating MDpsy.

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It should be noted that the SAL-treated SD (healthy control) and FSL-SIR (treatment-naïve control) groups presented in this manuscript were re-presented from Manuscript A. Data re-presented from Manuscript A are referred to in this manuscript as Mncube et al., 2020. The decision to separate this manuscript from Manuscript A was to present the work in a way that: 1) allowed for the manuscripts to be neatly separated into themes i.e. TRD and MDpsy; and 2) could be easily digested by the reader as opposed to a long, bulky, and convoluted paper. Further, the re-use of the aforementioned data was to prevent using a larger number of animals than necessary.

This manuscript focused on assessing the presence and treatment responsiveness or resistance of psychotic-like manifestations in the FSL-SIR model to OLZ and OFC. Hence, FLX treatment effects on psychotic-like manifestations (i.e. PPI) and the effects of OLZ and OFC on depressive-like behaviour (i.e. FST) are presented in Addendum B. This was based on the clinical approach to the diagnosis of psychotic symptoms either as SCZ or MDpsy. For this reason, depressive-like behaviour i.e. immobility in FST was not assessed in this manuscript. It is for this reason that the response of depressive-like behaviour to OLZ and OFC treatment are presented and discussed in Addendum B.

**Both co-authors have provided permission to submit this manuscript for examination as part of K Mncube's Ph.D thesis. The letters of consent are included in Addendum D.**

# Chapter 4: Manuscript B

## Bio-behavioural validation of a novel neurodevelopmental animal model of treatment resistant depression: Response to olanzapine with/without fluoxetine

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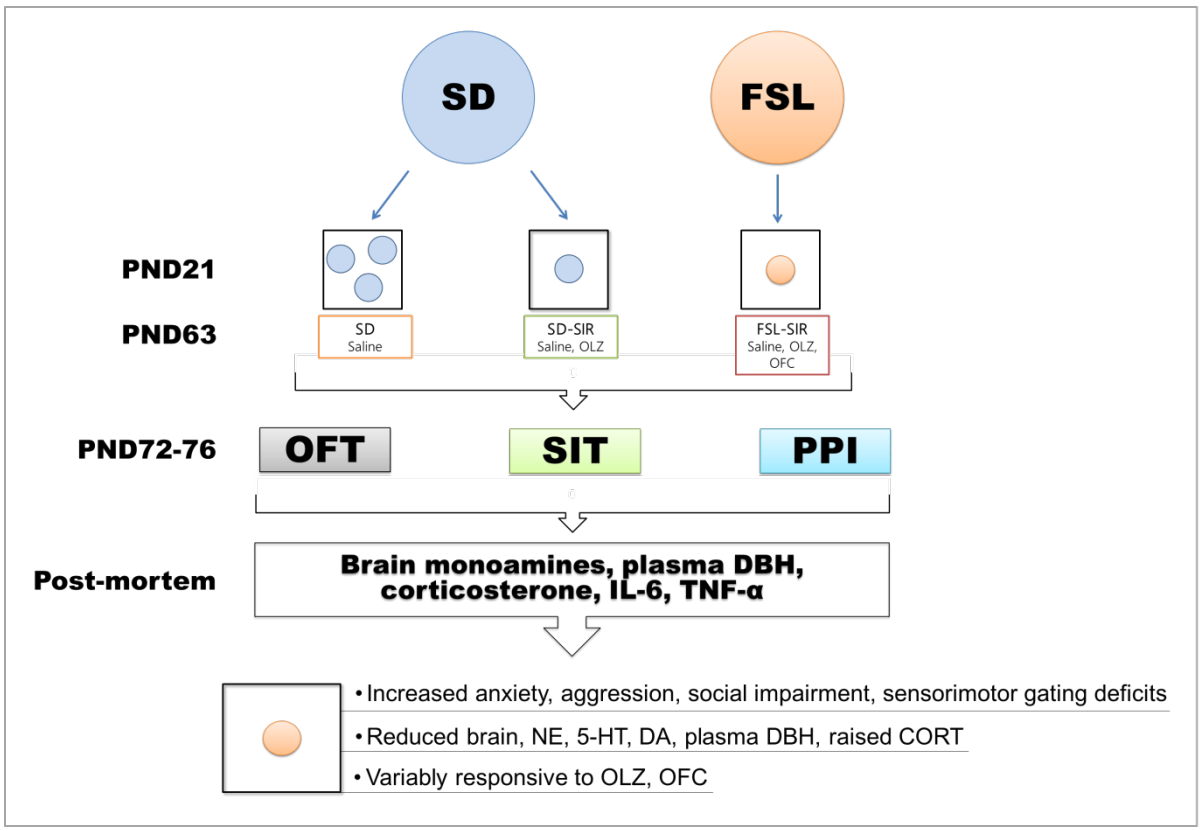
**Keywords:** gene-X-environment model; Flinder's Sensitive Line rat; social isolation rearing; olanzapine+fluoxetine; animal model; psychotic depression; prepulse inhibition test; bipolar disorder

# Chapter 4: Manuscript B

## Highlights

- Novel animal model of treatment resistant depression with co-occurring psychosis
- Exhibits significant thigmotactic behaviour variably responsive to OLZ and OFC
- Displays social withdrawal and social anxiety, resistant to OLZ and OFC
- Displays aggression and sensorimotor gating deficits most responsive to OFC
- Depleted NE, 5-HT, and DA, variably elevated by OLZ and OFC
- Reduced DBH, raised CORT and inflammation; treatment had anti-inflammatory effects

## Graphical abstract




## Abstract

Background: Psychosis in depression, or psychotic depression (MDpsy), contributes to the development of treatment resistant depression (TRD). Few animal models of TRD have been

studied with respect to psychotic-, social and aggressive-like behaviour and their responsive to an antidepressant/antipsychotic drug combination.

Methods: We deployed a gene-environment animal model of TRD. Sprague Dawley (SD) and Flinders Sensitive Line (FSL) pups were subjected to social or social isolation rearing (SIR) from weaning (postnatal day (PND) 21), receiving olanzapine (OLZ; 5 mg/kg x 14 days) or olanzapine+fluoxetine (5 mg/kg + 10 mg/kg for 14 days, only FSLs) from PND63, and compared to saline-treated SD controls. Psychotic-like, anxiety, and social behaviour were assessed from PND72. Post-mortem cortico-hippocampal norepinephrine (NE), serotonin (5-HT), and dopamine (DA), as well as plasma interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α), corticosterone (CORT), and dopamine-beta-hydroxylase (DBH) levels were evaluated.

Results: SD-SIR rats displayed sensorimotor gating deficits, anxiety, aggression, and disturbed cortico-hippocampal monoamines, all variably responsive to OLZ. SIR-exposed FSL rats exhibited significant sensorimotor gating deficits, anxiety, and social impairment, all responsive to OFC but not OLZ. In SIR-exposed FSL rats, OLZ and OFC reversed depleted NE, 5-HT, and DA, and decreased DBH and CORT.

Conclusion: SIR-exposed FSL rats display psychotic-like symptoms, social anxiety, and elevated CORT that are resistant to OLZ but variably responsive to OFC. Depleted cortico-hippocampal 5-HT, NE and DA were variably reversed by OLZ and OFC. Reduced DBH was unresponsive to treatment.

## 1. Introduction

Major depression (MD) has an estimated lifetime prevalence of 17% with up to a third of these patients developing treatment resistance to traditionally effective, first-line antidepressants (Cusin & Peyda, 2019; Rush *et al.*, 2004). Where there is underlying psychosis, otherwise known as psychotic depression (MDpsy), this further contributes to treatment-resistance (Fava, 2003; Nestler *et al.*, 2002; Schatzberg, 2003). MDpsy may have a similar or higher point prevalence as schizophrenia (SCZ) (Heslin & Young, 2018).

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MDpsy is diagnosed based on the occurrence of two or more major depressive episodes separated by an interval of at least two months (MD symptom free) *and* the occurrence of delusions and/or hallucinations that are either mood-congruent (guilt or sadness) or mood-incongruent (persecutory or paranoid) during MD episodes (APA, 2013; Tonna *et al.*, 2012). These patients typically suffer more intense depression, cognitive disturbances, and psychomotor agitation or retardation, and fare worse clinically than non-psychotically depressed patients (Cherian *et al.*, 2019; Keller *et al.*, 2007; Schatzberg, 2003). Other features of MDpsy include psychosocial difficulties; increased suicide; and a family history of mental disorder, especially bipolar disorder (BD) (Heslin & Young, 2018; Keller *et al.*, 2007). MD and MDpsy have been proposed to be distinct disorders due to the severity of MDpsy symptoms (Keller *et al.*, 2007; Rothschild, 2013), with MDpsy presenting with depressive symptoms similar to those of SCZ and psychotic bipolar disorder (PBD) (Jääskeläinen *et al.*, 2018; Keller *et al.*, 2007). Schizoaffective disorder and MDpsy are similar in syndromatic recovery (Jääskeläinen *et al.*, 2018). Compared to PBD, MDpsy is characterised by more severe negative symptoms, although both are similar with regard to rehospitalisation rates and functional outcome (Jääskeläinen *et al.*, 2018).

Antidepressant or antipsychotic monotherapy is inferior to combined antipsychotic-antidepressant treatment in treating MDpsy (Farahani & Correll, 2012). Co-therapy with olanzapine (OLZ) and fluoxetine (FLX) currently has the approval of the Food and Drug Administration (FDA) for the treatment of TRD (Caldarone *et al.*, 2015) but has proven useful in treating MDpsy (Rothschild, 2013). Mechanistically, the OLZ/FLX combination (OFC) increases extracellular levels of dopamine (DA), norepinephrine (NE) and serotonin (5-HT) in rat prefrontal cortex (Zhang *et al.*, 2000) superior to monotherapy with either agent. This multifunctional targeting is purported to mediate the beneficial effects of this combination, which dove-tails with the known biochemical anomalies of MDpsy (see below). Preclinically, OFC was shown to upregulate transcription factors that produce lasting changes in synaptic efficacy and improve cognitive performance (Horowitz *et al.*, 2003; Zhang *et al.*, 2000).

MDpsy is associated with cerebrospinal fluid and plasmic hyperdopaminergia, low dopamine-β-hydroxylase (DβH, DBH), and hypersecretion of cortisol (Schatzberg *et al.*, 1985); all of which are linked to psychosis and the pathophysiology of MDpsy (Schatzberg *et al.*, 2014; Schatzberg *et al.*, 1985). DBH, the enzyme catalysing the conversion of DA to NE, informs on central and peripheral

DA activity (Hamner & Gold, 1998), while reduced DBH is also associated with treatment resistance (Caldarone *et al.*, 2015). Neuroanatomically, the frontal cortex and hippocampus are implicated in depressive (Andrews *et al.*, 2015) and psychotic (Uys *et al.*, 2017) symptoms, with the prefrontal cortex implicated in cognitive function (Ott & Nieder, 2019) and mood regulation (Pandya *et al.*, 2012), and the hippocampus more involved in emotion, neuroendocrine stress hormone regulation and declarative memory (Nakahara *et al.*, 2018). Here, monoamines play a central role in the neurobiology and treatment of MD and MDpsy. In this regard, elevated NE (plasma) and 5-HT (platelets) have been associated with MDpsy and may differentiate it from MD (Goekoop *et al.*, 2012; Healy *et al.*, 1986).

Effective pharmacological management of MDpsy is challenging (Fava, 2003; Nestler *et al.*, 2002; Schatzberg, 2003). That said, part of the dilemma lies in the dearth of validated animal models of MDpsy to enable drug discovery initiatives. Some animal models demonstrate comorbid depression in SCZ (Samsom & Wong, 2015), while the ouabain model of BD attempts to address the co-occurrence of MD (manifested as hypo-activity) and mania (demonstrated by hyperactivity) (El-Mallakh *et al.*, 1995). However, no model accurately reproduces the co-occurrence of psychotic and depressive symptoms typical of MDpsy, while the lack of assessment of predictive validity is problematic.

Post-weaning social isolation rearing (SIR) is an early-life, psychosocial stressor that engenders late-life neurobiological and behavioural manifestations akin to psychosis (SCZ) (Moller *et al.*, 2015), anxiety (Rau *et al.*, 2015), and depression (Arndt *et al.*, 2015), including social deficits, aggression, and reduced sensorimotor gating (Forrest *et al.*, 2014). Early childhood adversity (modelled by SIR) may produce initial NE activation with increased DBH activity which is followed by chronic DBH suppression, similar to MDpsy (Hamner & Gold, 1998). The Flinders Sensitive Line (FSL) rat is a well-validated genetic model of MD (Overstreet & Wegener, 2013). Earlier work has prompted the idea of combining two translationally relevant rodent models to induce a TRD model (Brand & Harvey, 2017a, 2017b). Given the mood-psychosis continuum of MDpsy, such an approach holds great promise. That said, two recent studies have explored SIR in FSL rats (Bjornebekk *et al.*, 2007; Fischer *et al.*, 2012), although neither were designed to consider SCZ or MDpsy. However, a recent study (Mncube *et al.*, 2020) describes resistance to chronic fluoxetine treatment after exposing FSL rats to post-weaning SIR.

To further elaborate on the predictive validity of the above described model, and to address psychosis-related bio-behavioural characteristics, we have investigated locomotor activity, anxiety, social interaction and sensorimotor gating in SIR-exposed FSL rats following chronic OLZ or OFC treatment. In addition, cortico-hippocampal monoamines, plasma DBH, corticosterone, tumour necrosis factor (TNF)-α and interleukin (IL)-6 were analysed, the latter given the symptomatic overlap between MDpsy and SCZ and the causal role of inflammation in these disorders (Moller *et al.*, 2015).

## 2. Materials and Methods

### 2.1 Animals

This study was approved by the AnimCare animal research committee (NHREC reg. no. AREC-130913-015) of the North West University (NWU) (Ethics approval number: NWU-00150-18-S5). The animals used were bred, supplied and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform (PCDDP) at the NWU.

Male SD and FSL rats were used in this study. The original colonies of FSL rats were obtained from Dr David H Overstreet, University of North Carolina, USA. The effects of SIR on increased anxiety and hyperactivity are not consistently observed in female rats (Walker *et al.*, 2019; Weiss *et al.*, 2004) and because this is a requirement for the proposed model, female rats were not used in the study. All animals were exposed to the same olfactory, visual, and auditory cues, although FSL-SIR rats were deprived of social contact with peer rats during this period. All rats were allowed free access to standard laboratory chow and water, and housed in identical transparent cages (380 mm x 380 mm x 230 mm) in an environmentally-controlled room: constant temperature (22 ± 4°C), humidity (50 ± 20%), and a 12:12 hour light-dark cycle (lights on 06:00) with no to minimal noise.

### 2.2 Study design

The present study formed part of a larger study in which we sought to develop a validated animal model of major depression with psychotic features, based on exposing FSL rats to post-weaning SIR. In a parallel study (Mncube *et al.*, 2020) we demonstrated more pronounced depressive

symptoms in FSL-SIR rats together with resistance to a standard of care antidepressant: FLX. To avoid unnecessary use of animals, the present study uses the same SAL-treated control animals, namely, SD and FSL-SIR rats, to demonstrate psychotic-like and enhanced social anxiety and aggressive manifestations as well as pathological deviations in FSL-SIR biochemistry and their response to OLZ and OFC treatments. Combining data from the two studies into a single paper proved bulky and unwieldy. Therefore, in the interest of disseminating the knowledge in the best way possible without wastage of animals (in agreement with the "refine, reduce, and replace" principle (Singh, 2012)), we opted to present the data as two separate manuscripts albeit having the same objective.

The study design is presented in Figure 1. Animals were weaned on post-natal day (PND) 21. FSL rats are derived from the SD strain, with either SD or Flinders Resistant Line (FRL) rats used as control animals (Overstreet and Wegener, 2013). SD rats were assigned to social-rearing (SD, 3 rats/cage) or social isolation rearing (SIR, 1 rat/cage) while the FSL rats were reared in social isolation. Rearing conditions were maintained for a period of 8 weeks (Moller *et al.*, 2013; Uys *et al.*, 2016). At PND63, while remaining in their assigned rearing condition, SD-SIR and FSL-SIR animals were assigned to a treatment group: saline-treated (SAL) or OLZ-treated. Socially-reared SD rats received only SAL. Thus, the resultant cohorts were as follows: SD-SAL, SD-SIR-SAL, SD-SIR-OLZ, FSL-SIR-SAL, and FSL-SIR-OLZ. An additional treatment group for FSL-SIR-OFC was added to determine the effects of a combination of FLX and OLZ (OFC) on behaviour and neurochemistry. Each cohort contained 12 rats (n = 12 per cohort) and a total of 72 animals was used in this study. The animals were first weighed on the day of weaning and then again each morning from the beginning of the treatment protocol (PND 63) until the last day of the study (PND 77). Their weights were used to calculate the volume of drug to be administered and to ensure animals across the treatment groups showed equal growth. The treatment regimen commenced from PND 63 and continued until PND76. Behavioural testing commenced on PND 72 beginning with the OFT, followed by the social interaction test (SIT) on PND74, and the prepulse inhibition test (PPI) on PND76. The animals were euthanised by decapitation without prior administration of an anaesthetic. Trunk blood and brain tissue was collected for bioanalysis. For behavioural and monoamine analysis, all animals (n = 12 per cohort) were included in the data. For ELISA analysis, ten (n = 10 per cohort) plasma samples were randomly selected from the 12 animals per cohort. This was to allow for more samples to be assayed per plate while maintaining statistical power.

DBH, CORT, IL-6, and TNF-α were quantified in plasma rather than the brain to correlate to clinical findings which are mainly based on fluid sample readouts.

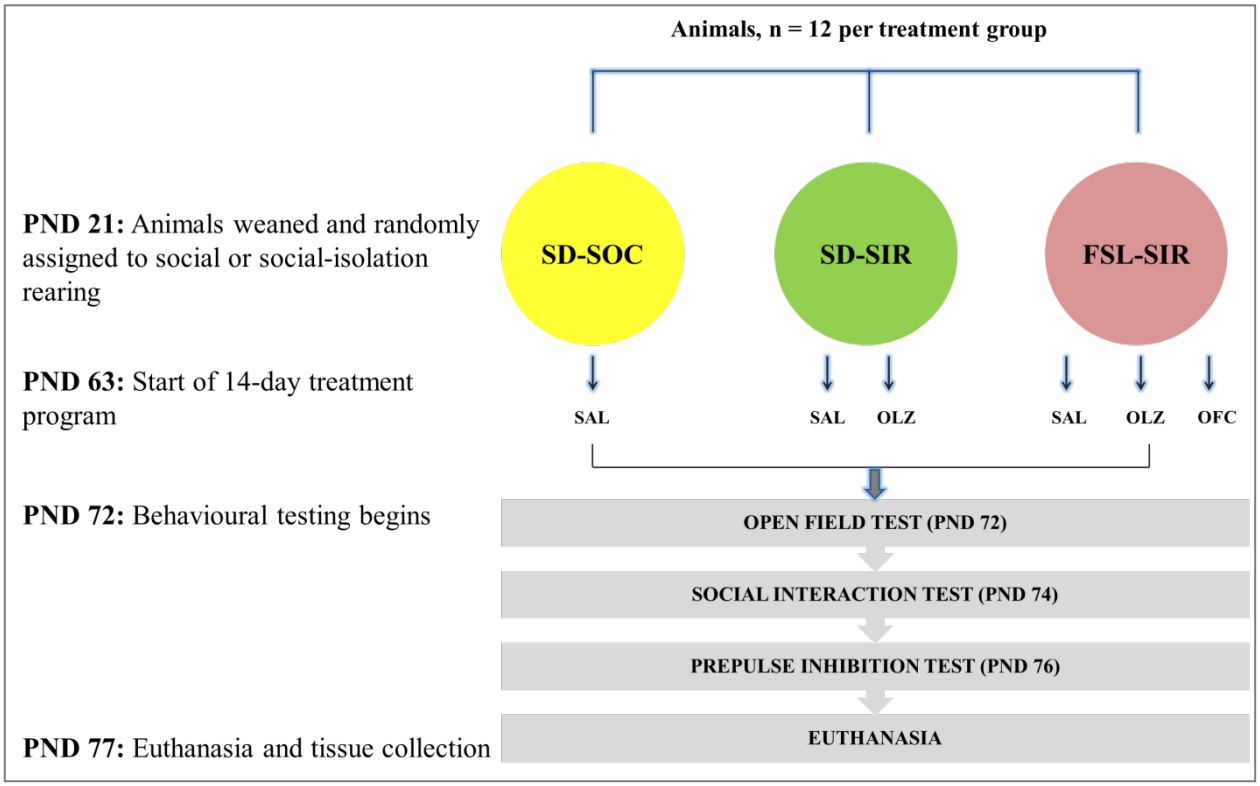


**Figure 1:** Study design

### 2.3 Drug preparation and treatment protocol

FLX (fluoxetine hydrochloride; Pubchem CID 62857; Jade Pharmaceuticals, South Africa) was first dissolved in approximately 500 µL distilled water and then made up to 10 mg/kg in physiological saline. OLZ (olanzapine; Pubchem CID 135398745; DB Fine Chemicals (Pty) Ltd (Johannesburg, South Africa) was dissolved in approximately 200 µL 0.1 N acetic acid (Pubchem CID 176) and then in saline to make a 5 mg/kg solution. All treatments were administered subcutaneously (s.c) (Zhang *et al.*, 2000) according to the literature as follows: 10 mg/kg FLX (Detke *et al.*, 1995), OLZ (5 mg/kg) (Heidbreder *et al.*, 2001) and OFC (5 mg/kg + OLZ 10 mg/kg FLX) for a period of 14 days. Control rats received SAL s.c. All treatments were administered during the light cycle between 08:00 and 10:00.

### 2.4 Behavioural assessments

The behavioural experiments are presented in the sequence in which they were performed. That is, from least to most stressful as described by Mokoena *et al.* (2015) to ensure that that the behaviour in subsequent tests are not negatively affected by prior tests.

#### 2.4.1 Psychosis: Hyperlocomotion – OFT

Spontaneous hyperactivity in rats in response to novel environments is useful for assessing psychomotor agitation (a symptom of SCZ and sometimes seen in MDpsy) (Moller *et al.*, 2015; Schatzberg & Rothschild, 1992). The method of Sherif and Oreland (1995) was used to determine the total distance travelled (cm) in the OFT. Rats were placed individually into the centre of a square arena (100 x 100 x 50 cm). The test was conducted in a dimly lit room illuminated with red light (40 W). Animal behaviour was recorded for 5 min using a ceiling-mounted digital camera. The video files were analysed using Noldus Ethovision XT software (Noldus® Information Technology, Wageningen, The Netherlands).

#### 2.4.2 Anxiety: Thigmotaxis – OFT

Thigmotaxis is an important indicator of anxiety in rodents and is sensitive to treatment with anxiolytics and sedatives (Belovicova *et al.*, 2017). The ratio of time (s) spent in the centre of the arena versus the time (s) spent along the walls of the arena (presented as a percentage) were be used to determine relative anxiety levels in the test subjects. Behaviour was recorded within the first 5 min which gives better insight into anxious behaviour (Gould *et al.*, 2009).

#### 2.4.3 Social Interaction Test (SIT)

Social deficits, anxiety as well as aggression are recognised symptoms of MDpsy (Keller *et al.*, 2007; Tyrka *et al.*, 2006). The SIT was performed in the same arena and under the same lighting conditions as in the OFT and as previously described (Moller *et al.*, 2011) to assess anxiety-related social withdrawal and antisocial behaviour in rodents (File & Seth, 2003; Kaidanovich-Beilin *et al.*, 2011). Pair scores were used and are expressed as percentage (%). The behaviours assessed are described in Table 1. For brevity, they are presented in the data as "Social (amicable)", "Asocial (anxiety-like)," and "Aggressive (antisocial)" behaviour.

Table 1: Behaviour scored in the social interaction test (Adapted from Barnett (1958), Blanchard and Blanchard (1977), and Brain *et al.* (1989).

| Category | Behaviour | Description |
|---|---|---|
| Social | Sniffing | Sniffing the head, snout, anogenital area, or body of the partner |
| | Approaching | Walking directly toward the partner |
| | Following | Moving in close proximity to the partner as it walks around the arena |
| | Grooming (allo-grooming) | Grooming the body of the partner using the mouth |
| | Crawling over/under | Both forepaws placed on the partner, with the head and anterior part of the body pushed underneath the partner |
| Asocial (anxiety) | Exploring | Walking or running around the arena, not obviously directed toward the partner, supported or unsupported rearing unrelated to partner |
| | Freezing | Complete immobility, no movement of any part of the body, with all four paws on the arena |
| | Self-grooming | Licking the fur on the flanks or abdomen or preening the tail |
| | Evading | Movement of the head and/or body away from the partner |
| Antisocial (aggression) | Nose-off | Defensive or offensive strategy in which partners stand immobile facing each other; may escalate to boxing |
| | Boxing | Two rats stand on their hind legs facing each other and push and paw at each other using front paws |

### 2.4.4 Psychosis: Sensorimotor gating – prepulse inhibition (PPI) test

PPI is used to determine sensorimotor gating performance in humans and rodents (Shoji & Miyakawa, 2018), deficits of which correlate with clinical symptoms of disordered thoughts and distractibility (Forrest *et al.*, 2014) evident in psychosis (APA, 2013). PPI was assessed in two

ventilated and illuminated, sound-attenuating startle chambers (SR-LAB, San Diego Instruments, San Diego, USA), as described previously (Moller *et al.*, 2011).

Per cent PPI (%PPI) for each prepulse+pulse trial was calculated using the following formula: %PPI=[100−(startle response for PREPULSE+PULSE trial)/(startle response for PULSE ALONE trial)×100] (Swanepoel *et al.*, 2018). Average %PPI values across the four prepulse intensities were calculated and used.

### 2.5 Bioanalysis

#### 2.5.1 Preparation of plasma and brain tissue

The frontal cortex and hippocampus were excised on an ice-cooled glass slab immediately after decapitation as previously described (Mokoena *et al.*, 2015; Möller *et al.*, 2013). Trunk blood was collected in pre-chilled, 4 mL vacutainer tubes (Vacuette®) containing $K_3$EDTA solution as anti-coagulant. The blood was centrifuged at 1000 × g at 4°C for 15 min. Brain tissue and plasma were fixed in liquid nitrogen and stored at −80°C until the day of analysis.

#### 2.5.2 Monoamine quantification

NE, 5-HT, and DA were quantified in the selected brain regions using a high-performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (Viljoen *et al.*, 2018). An Agilent 1200 series HPLC (Agilent Technologies Inc., Santa Clara, CA USA), equipped with an isocratic pump and autosampler, coupled to an ESA Coulochem III Electrochemical detector with a coulometric flow cell (Model 5011A High Analytical Cell and Guard cell 5020) and Chromeleon® Chromatography Management System version 6.8 (obtained from Thermo Fisher Scientific, Waltham, MA USA), was used for this analysis.

#### 2.5.3 Psychosis biomarkers: plasma DBH, inflammatory cytokines and CORT

DBH (Abbexa, Cambridge, UK), IL-6 and TNF-α (Elabscience, Wuhan, China), and CORT (Elabscience, Wuhan, China) were measured in the plasma by sandwich ELISA kits according to the manufacturer's protocol. For each ELISA kit, a total of 10 plasma samples from each cohort was analysed.

### 2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism® 8 for Windows (GraphPad Software Inc., San Diego, CA, USA) under the supervision of the Statistical Consultation Service of the NWU. All data were checked for normality using Shapiro-Wilk's test. Two-way ANOVA and Bonferroni post-hoc test was applied in comparisons of body weight, behaviour, and biochemistry. Significance was set at $p < .05$ for all comparisons. If no statistical significance was indicated in the post-hoc test, Cohen's $d$ value was calculated to establish the effect size and practical significance. Large effect sizes are indicated by $d \geq 0.8$ and very large effect sizes by $d \geq 1.2$ (Sawilowsky, 2009). Only these effect sizes are noted in the figures and discussed. Data are graphically presented as mean ± SEM.

## 3. Results

To confirm the translational relevance of the FSL-SIR rat for treatment resistance as well as anomalous behaviour and neurochemistry, control data relating to bio-behavioural comparisons between SAL-treated SD and FSL-SIR animals have been re-presented from our earlier study (Mncube et al., 2020, submitted), as a healthy control (SD) and a treatment-naïve control (FSL-SIR).

### 3.1 Body weight

A two-way ANOVA of body weight revealed significant main effects of treatment [$F(13, 924) = 38.37$, $p < .0001$] and strain [$F(5, 924) = 264.7$, $p < .0001$] (data not shown). No interaction between these two factors was noted. SAL-treated SD-SIR and FSL-SIR; OLZ-treated SD-SIR and FSL-SIR; as well as OFC-treated FSL-SIR were all significantly heavier than SD-SAL rats ($p < .0001$). SAL-treated FSL-SIR rats were significantly heavier than SAL-treated SD-SIR ($p < .0001$). OLZ-treated SD-SIR ($p = .0498$), OLZ-treated FSL-SIR ($p < .0001$), and OFC-treated FSL-SIR ($p < .0001$) animals weighed significantly less than SAL-treated SD-SIR animals. FSL-SIR-SAL weighed significantly more than SD-SIR-OLZ rats ($p < .0001$) while OLZ-treated FSL-SIR ($p < .0001$) and OFC-treated FSL-SIR ($p = .0003$) rats weighed significantly less than OLZ-treated SD-SIR rats. OLZ-treated and OFC-treated FSL-SIR rats weighed significantly less than SAL-treated FSL-SIR animals (both $p < .0001$). OFC-treated FSL-SIR animals weighed significantly more than OLZ- treated FSL-SIR ($p < .0001$). Overall rate of weight gain in SIR exposed FSL rats was more rapid than SD-SIR animals (both SAL-treated; 7.3% vs. 6.9%). When these groups were treated with OLZ, FSL-SIR animals lagged behind SD-SIR

animals in gaining weight (6.2% vs. 7.0%). OFC-treated FSL-SIR animals gained weight faster than FSL-SIR-OLZ (6.5% vs. 6.2%).

### 3.2 OFT

*Locomotor activity*. A significant main effect of strain [$F(5, 55) = 7.067$, $p < .0001$] on distance travelled but no significant main effect of treatment or strain x treatment interaction was noted. Distance travelled is shown in Figure 2A. OFC-treated FSL-SIR animals travelled significantly less than SD-SAL rats ($p = .0002$), SAL-treated SD-SIR ($p = .0001$), OLZ-treated SD-SIR ($p = .0008$), and SAL-treated FSL-SIR ($p = .0033$). Cohen's $d$ analysis showed a large effect size decrease in locomotor activity in FSL-SIR rats following OFC treatment and compared to FSL-SIR rats following OLZ treatment ($d = 1.0$), and in OLZ-treated FSL-SIR compared to SD-SAL rats and SD-SIR-SAL (both $d = 1.0$).

*Thigmotaxis*. A significant main effect of strain [$F(5, 55) = 4.541$, $p = .0015$] with no main effect of treatment or strain x treatment interaction was indicated. SIR of SD and FSL rats increased thigmotaxis versus SD-SAL controls (Figure 2B), however, the increase was more pronounced in FSL-SIR rats compared to the aforementioned controls. FSL-SIR-SAL rats exhibited significantly more thigmotactic behaviour than SD-SAL ($p = .0034$) and SD-SIR-OLZ rats ($p = .0029$). OFC significantly reduced thigmotactic behaviour in FSL-SIR rats compared to SAL-treatment ($p = .0122$). Cohen's $d$ analysis showed a large effect size increase in thigmotactic behaviour by SD-SIR-SAL rats versus SD-SAL ($d = 0.8$) and in FSL-SIR-SAL compared to SD-SIR-SAL ($d = 0.8$) and a very large effect size increase compared to FSL-SIR-OLZ ($d = 1.2$).

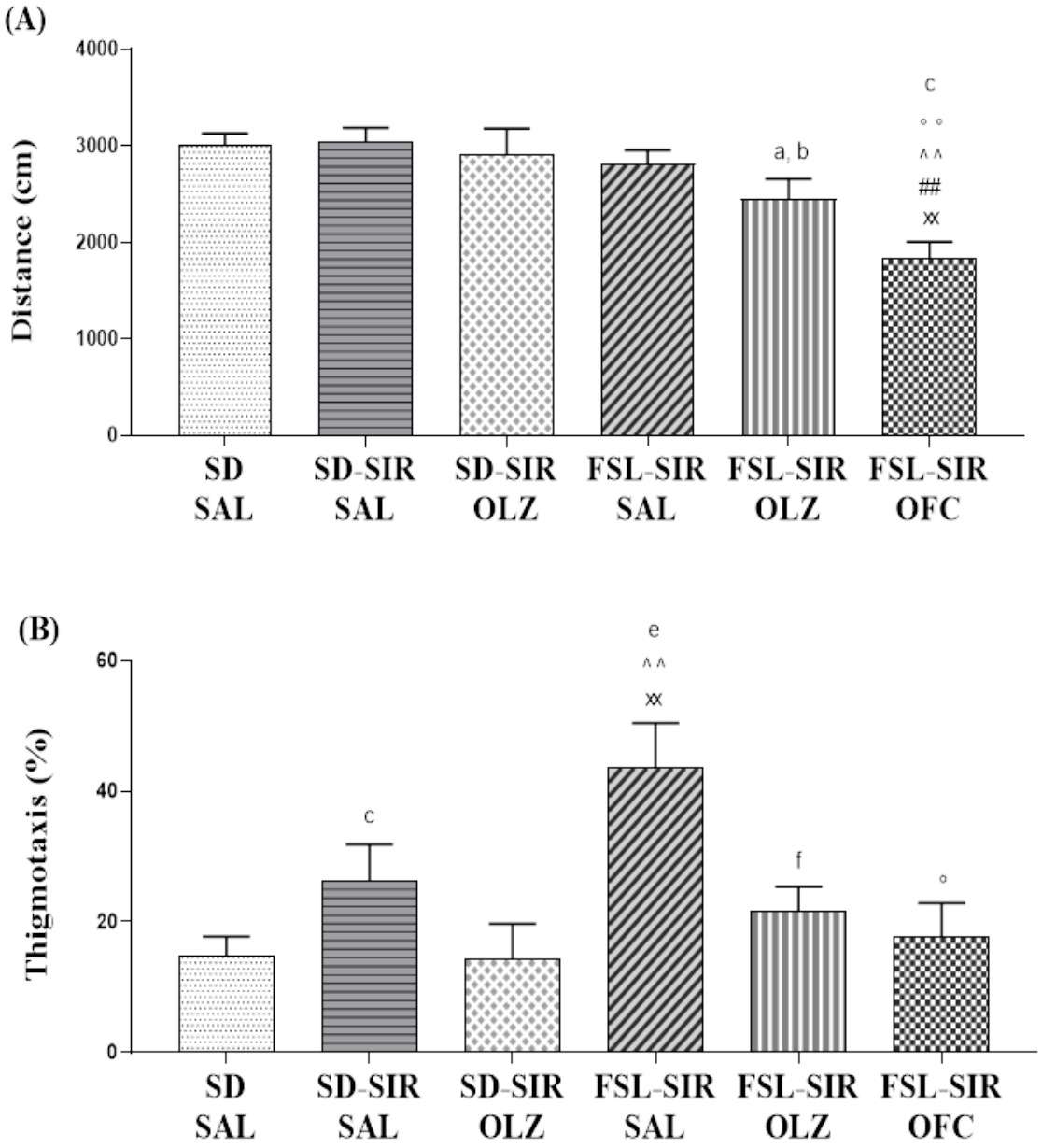


**Figure 2A.** Locomotor activity in SD, SD-SIR, and FSL-SIR rats as measured in the OFT (n = 12/group). $^{xx}p$ < .01 vs. SD-SAL; $^{\#\#}p$ < .01 vs. SD-SIR-SAL; $^{\wedge\wedge}p$ < .01 vs. SD-SIR-OLZ; $^{\circ\circ}p$ < .01 vs. FSL-SIR-SAL; $^{a}d$ = 1.0 vs. SD; $^{b}d$ = 1.0 vs. SD-SIR; $^{c}d$ = 1.0 vs. FSL-SIR-OLZ. **Figure 2B:** Thigmotaxis in SD, SD-SIR, and FSL-SIR rats as measured in the OFT (n = 12/group). $^{xx}p$ < .01 vs. SD-SAL; $^{\wedge\wedge}p$ < .01 vs. SD-SIR-OLZ; $^{\circ}p$ < .05 vs. FSL-SIR-SAL; $^{c}d$ = 0.8 vs. SD-SAL; $^{e}d$ = 0.8 vs. SD-SIR-SAL; $^{f}d$ = 1.2 vs. FSL-SAL-SIR. For figures 2A and 2B, SD-SAL and FSL-SIR-SAL are re-presented from Mncube et al., 2020. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; OLZ, olanzapine, OFC, olanzapine+fluoxetine.

### 3.3 SIT

*Social (amicable)*. Significant main effects of strain [$F(5, 175) = 5.741$, $p < .0001$] and treatment [$F(35, 175) = 15.89$, $p < .0001$] were revealed with no strain x treatment interaction. Although SD-SIR-SAL rats were not significantly different from SD-SAL controls, combining FSL and SIR evoked a decrease in this behaviour (Figure 3A). SAL-treated FSL-SIR rats were significantly less social than healthy SD-SAL controls ($p = .0023$), SAL-treated SD-SIR ($p = .0142$), and OLZ-treated SD-SIR ($p < .0001$).

*Asocial (social anxiety-like)*. Significant main effects of treatment [$F(17, 85) = 57.67$, $p < .0001$] and strain [$F(5, 85) = 6.015$, $p < .0001$] were noted without strain x treatment interaction. SD-SIR-SAL rats were not significantly different from SD-SAL controls; however, combining FSL and SIR evoked an increase in this behaviour (Figure 3B). Significantly increased asocial behaviour was exhibited by SAL-treatment FSL-SIR animals compared to SD-SAL ($p = .0107$), SD-SIR-SAL rats ($p = .0235$), and SD-SIR-OLZ ($p < .0001$). OLZ-treated FSL-SIR rats were more asocial with their partners than OLZ-treated SD-SIR animals ($p = .0335$).

*Aggression (antisocial)*. No strain x treatment interaction and no main effects of strain or treatment were indicated. Although SD-SIR-SAL rats were not significantly different from SD-SAL controls, combining FSL with SIR evoked an increase in this behaviour (Figure 3C). Cohen's *d* analysis showed a large effect size increase in aggressive behaviour in FSL-SIR-SAL ($d = 1.0$) and FSL-SIR-OLZ ($d = 1.0$) animals compared to SD-SAL rats. OLZ decreased aggression in FSL-SIR rats with a large effect size of $d = 0.8$ compared to SAL-treatment. OFC treatment caused a large effect size reduction in aggressive behaviour in FSL-SIR rats compared to SAL-treated FSL-SIR animals ($d = 1.0$) and very large effect size reduction compared to OLZ-treated FSL-SIR animals ($d = 1.2$).

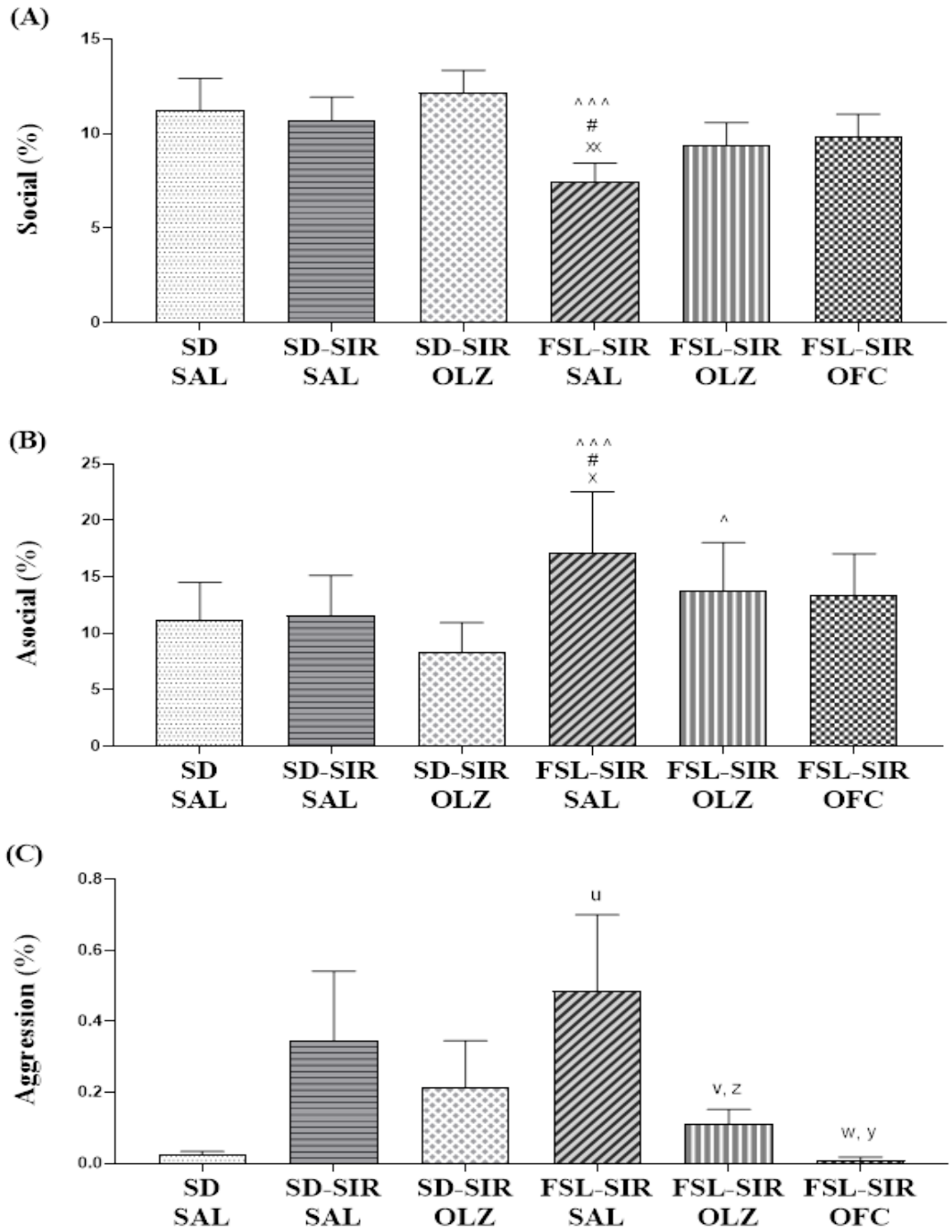


**Figure 3:** Behaviour of SAL- and drug-treated SD, SD-SIR, and FSL-SIR rats as measured in the SIT (n = 12/group). **(A)** Social/amicable behaviour. $^{xx}p < .01$ vs. SD-SAL; $^{\#}p < .05$ vs. SD-SIR-SAL; $^{\wedge\wedge\wedge}p < .0001$ vs. SD-SIR-OLZ. **(B)** Asocial/socially anxious-like behaviour. $^{x}p < .05$ vs. SD-SAL; $^{\#}p < .01$ vs. SD-SIR-SAL; $^{\wedge\wedge\wedge}p < .0001$, $^{\wedge}p < .05$ vs. SD-SIR-OLZ. **(C)** Antisocial/aggressive behaviour. $^{u}d = 1.0$, $^{v}d = 1.0$ vs. SD; $^{z}d = 0.8$; $^{w}d = 1.0$ vs. FSL-SIR-SAL; $^{y}d = 1.2$ vs. FSL-SIR-OLZ. For figures 3A and 3B, SD-SAL and FSL-SIR-SAL are re-presented from Mncube et al., (2020). Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; OLZ, olanzapine, OFC, olanzapine+fluoxetine.

### 3.4 Sensorimotor gating

*Acoustic startle response (ASR).* A two-way ANOVA showed significant main effects of BLOCK [$F(3, 15) = 15.41$, $p < .0001$] and treatment [$F(5, 15) = 9.0$, $p = .0004$] with no BLOCK x treatment interaction. Startle response was highest in the first block across all cohorts; thereafter, in-session habituation was observed. Across all BLOCKs, significant startle suppression was observed in FSL-SIR-SAL ($p = .0067$), FSL-SIR-OLZ ($p = .0009$), and FSL-SIR-OFC ($p = .0010$) compared to SD-SAL animals (data not shown).

*Average % PPI.* A significant main effect of strain [$F(5, 55) = 4.265$, $p = .0024$] with no main effect of treatment or strain x treatment interaction was evident. Figure 4 shows significantly reduced %PPI in SD-SIR-SAL ($p = .0086$), SD-SIR-OLZ ($p = .0058$), FSL-SIR-SAL ($p = .0132$), and FSL-SIR-OLZ ($p = .0115$) animals compared to SD-SAL animals. A very large effect size reduction in %PPI was observed in OFC-treated FSL-SIR rats compared to SD-SAL rats ($d = 1.3$).

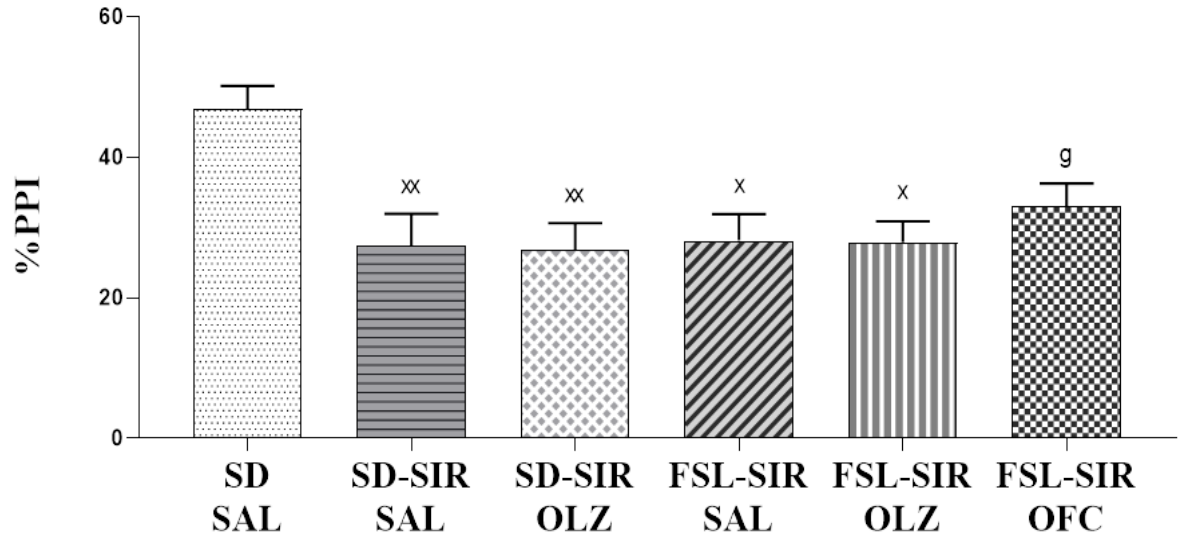


**Figure 4:** Average %PPI in SAL- and drug-treated SD, SD-SIR, and FSL-SIR rats (n = 12/group). $^{xx}p < .01$, $^{x}p < .05$ vs. SD-SAL; $^{g}d = 1.3$ vs. SD-SAL. SD-SAL and FSL-SIR-SAL are re-presented from Mncube et al., (2020). Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; OLZ, olanzapine, OFC, olanzapine+fluoxetine.

### 3.5 Monoamines

*NE: Frontal cortex* (Figure 5A). A significant main effect of strain [$F(5, 55) = 42.52$, $p < .0001$] but no effect of treatment or an interaction between the two were indicated. SD-SIR-SAL rats presented with reduced NE levels versus SD-SAL controls, while SIR of FSL rats evoked an even greater decrease, with the latter reversed by OLZ or OFC. FSL-SIR-SAL, FSL-SIR-OLZ (both $p < .0001$), and FSL-SIR-OFC ($p = .0007$) presented with significantly reduced frontocortical NE compared to SD-SAL rats. Significantly reduced NE in this region was observed in FSL-SIR-SAL ($p < .0001$) and FSL-SIR-OLZ ($p = .0077$) rats compared to SAL-treated SD-SIR rats. FSL-SIR-SAL ($p < .0001$), FSL-SIR-OLZ ($p = .0002$), and FSL-SIR-OFC ($p = .0199$) presented with significantly reduced frontocortical NE compared to OLZ-treated SD-SIR rats. OLZ and OFC treatment significantly elevated NE in this region in FSL-SIR rats (both $p < .0001$) compared to SAL-treated FSL-SIR animals. Cohen's *d* analysis showed a large effect size reduction in SD-SIR-SAL NE compared to SD-SAL ($d = 1.0$).

*Hippocampus* (Figure 5B). A significant main effect of strain [$F(5, 55) = 25.84$, $p < .0001$] with no effect of treatment or strain x treatment interaction was evident. SD-SIR-SAL rats had reduced NE levels versus SD-SAL controls, while combining FSL and SIR evoked an even greater decrease, with the latter reversed by OLZ or OFC. Cohen's *d* showed a large effect size decrease in hippocampal NE in SD-SIR-SAL compared to SD-SAL ($d = 1.0$), which was reversed by OLZ by a large effect size compared to SD-SAL rats ($d = 1.0$). Significantly reduced hippocampal NE was observed in FSL-SIR-SAL ($p < .0001$), FSL-SIR-OLZ ($p = .0009$), and FSL-SIR-OFC ($p = .0043$) compared to SD-SAL rats. SAL-treated FSL-SIR animals had significantly diminished NE in this region compared to SAL-treated SD-SIR ($p < .0001$). SAL- and OLZ-treated FSL-SIR (both $p < .0001$) and OFC-treated FSL-SIR animals ($p = .0004$) presented with significantly reduced hippocampal NE compared to OLZ-treated SD-SIR. OLZ ($p = .0002$) and OFC ($p < .0001$) treatment significantly increased NE in FSL-SIR compared to SAL-treated animals.

*5-HT: Frontal cortex* (Figure 5C). A significant main effect of strain [$F(5, 55) = 5.415$, $p = .0004$] was observed with no main effect of treatment and no interaction between these factors. SD-SIR-SAL rats had increased 5-HT levels versus SD-SAL controls, FSL rats exposed to SIR evoked the opposite response, with the latter reversed by OLZ or OFC. Cohen's *d* showed a large effect size elevation of frontocortical 5-HT in SD-SIR-SAL compared to SD-SAL ($d = 1.1$). FSL-SIR-SAL rats also presented with a very large effect size reduction in 5-HT compared to SD-SAL rats ($d = 4.4$). FSL-SIR-SAL rats

presented with significantly reduced 5-HT in this region compared to SD-SIR-SAL ($p$ = .0033). Significant elevations were measured in FSL-SIR rats following OLZ ($p$ = .0270) and OFC ($p$ = .0002) treatment compared to SD-SAL-treatment. A very large effect size decrease in 5-HT followed OLZ treatment in SD-SIR rats compared to SAL treatment ($d$ = 1.7). FSL-SIR-SAL rats presented with a very large effect size reduction in 5-HT compared to SD-SIR-OLZ ($d$ = 3.2). OLZ caused a large effect size increase of 5-HT in FSL-SIR rats compared to OLZ-treated SD-SIR rats ($d$ = 0.8).

*Hippocampus* (Figure 5D). A significant main effect of strain [$F(5, 55) = 11.40$, $p < .0001$] was noted with no effect of treatment and no strain x treatment interaction. SD-SIR-SAL rats had increased 5-HT levels versus SD-SAL controls, while SIR of FSL rats evoked the opposite response, the latter reversed by OLZ or OFC. Cohen's $d$ analysis showed a large effect size elevation in hippocampal 5-HT in SD-SIR-SAL compared to SD-SAL rats ($d$ = 0.8). SAL-treated FSL-SIR rats were found to have significantly reduced 5-HT in this region compared to SD-SAL and SD-SIR-SAL (both $p < .0001$), and SD-SIR-OLZ ($p$ = .0017) rats. OFC treatment significantly raised hippocampal 5-HT in the FSL-SIR compared to SAL treatment ($p < .0001$). 5-HT was still significantly reduced in FSL-SIR rats following OLZ treatment compared to SAL-treated SD-SIR ($p$ = .0022). A very large effect size reduction in SD-SIR rat hippocampi followed OLZ treatment compared to SAL treatment ($d$ = 1.4). OLZ treatment caused a very large effect size elevation in hippocampal 5-HT in FSL-SIR compared to SAL-treatment ($d$ = 2.0).

*DA*: *Frontal cortex* (Figure 5E). No strain x treatment interaction and no main effects of strain or treatment were indicated. Although SD-SIR-SAL rats were not significantly different from SD-SAL controls, combining FSL and SIR evoked a decrease in DA, the latter reversed by OLZ or OFC. Cohen's $d$ showed a large effect size decrease in SAL-treated FSL-SIR rats compared to SD-SAL ($d$ = 0.8) and a large effect size elevation in OLZ-treated ($d$ = 0.9) and OFC-treated ($d$ = 1.1) FSL-SIR rats compared to FSL-SIR-SAL rats.

*Hippocampus* (Figure 5F). A significant main effect of treatment [$F(11, 55) = 4.948$, $p < .0001$] but none of strain and no interaction between the two was observed. Although SD-SIR-SAL rats were not significantly different from SD-SAL controls, SIR of FSL rats evoked a decrease in DA, the latter reversed by OLZ and OFC. OFC significantly elevated DA in FSL-SIR compared to SAL-treatment ($p$ = .0470). Cohen's $d$ showed a large effect size reduction in DA in FSL-SIR-SAL rats compared to SD-SAL ($d$ = 1.2) and very large effect size reduction versus SD-SIR-SAL ($d$ = 1.3). A large effect size

elevation in DA in FSL-SIR rats followed OLZ treatment compared to SAL-treated FSL-SIR ($d$ = 1.0) and compared to SD-SIR-OLZ ($d$ = 1.0) rats.

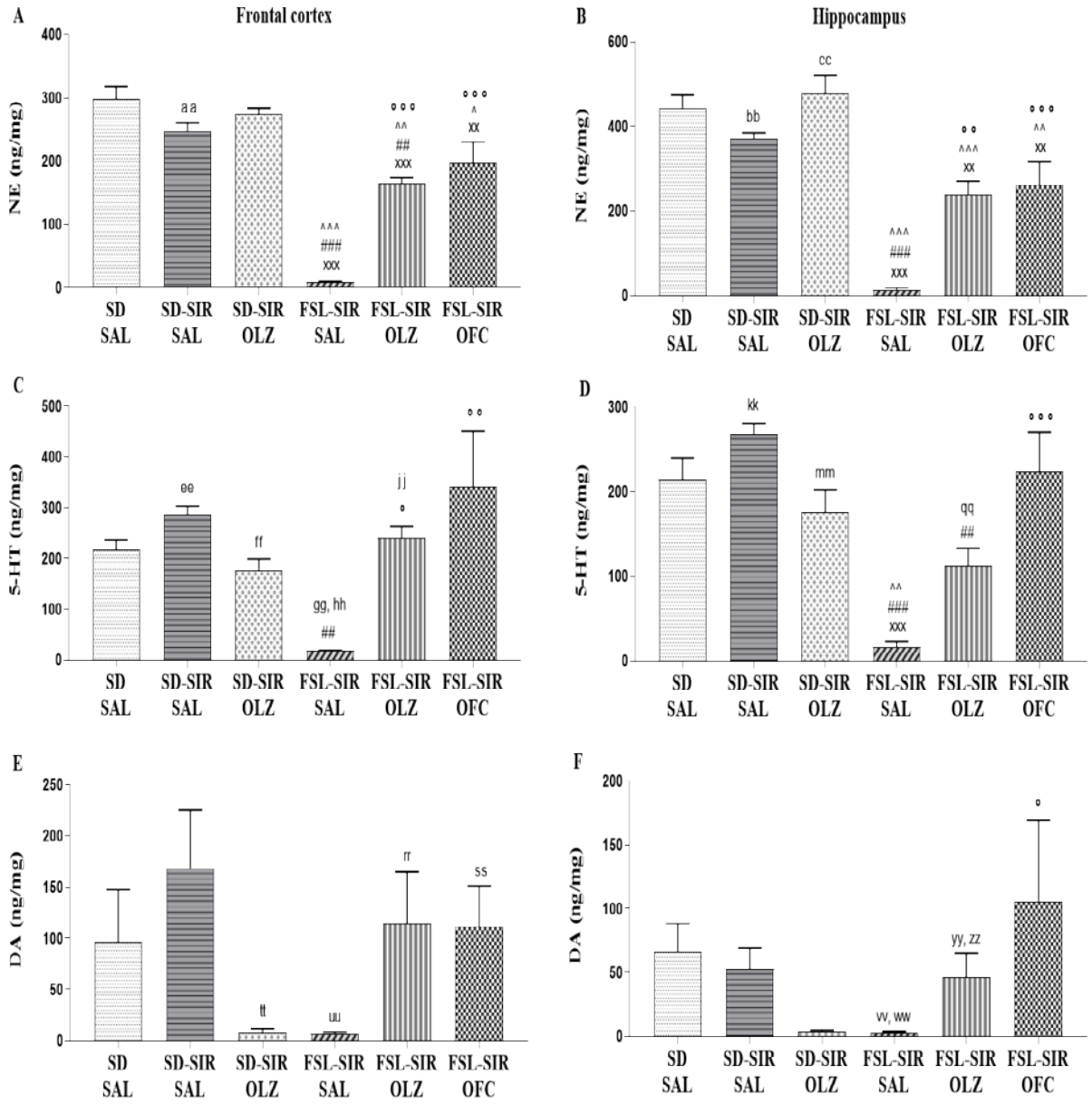


**Figure 5:** Monoamine levels in the frontal cortex (A, C, E) and hippocampus (B, D, F) in SAL- and drug-treated SD, SD-SIR, and FSL-SIR rats (n = 12/group). **(A)** NE. $^{xxx}p < .0001$, $^{xx}p < .01$ vs. SD-SAL; $^{###}p < .0001$, $^{##}p < .01$ vs. SD-SIR-SAL; $^{\wedge\wedge\wedge}p < .0001$, $^{\wedge\wedge}p < .01$, $^{\wedge}p < .05$ vs. SD-SIR-OLZ; $^{\circ\circ\circ}p < .0001$ vs. FSL-SIR-SAL; $^{aa}d$ = 1.0 vs. SD-SAL. **(B)** NE. $^{xxx}p < .0001$, $^{xx}p < .01$ vs. SD-SAL; $^{###}p < .0001$ vs. SD-SIR-SAL; $^{\wedge\wedge\wedge}p < .0001$, $^{\wedge\wedge}p < .01$ vs. SD-SIR-OLZ; $^{\circ\circ\circ}p < .0001$, $^{\circ\circ}p < .01$ vs. FSL-SIR-SAL; $^{bb}d$ = 1.0 vs. SD-SAL; $^{cc}d$ = 1.0 vs. SD-SIR-SAL. **(C)** 5-HT. $^{##}p < .01$ vs. SD-SIR-SAL; $^{\circ\circ}p < .01$, $^{\circ}p < .05$ vs. FSL-SIR-SAL $^{ee}d$ = 1.1, $^{gg}d$ = 4.4 vs. SD-SAL; $^{ff}d$ = 1.7 vs.SD-SIR-SAL; $^{hh}d$ = 3.2, $^{jj}d$ = 0.8 vs. SD-SIR-OLZ. **(D)** 5-HT. $^{xxx}p < .0001$ vs. SD-SAL; $^{###}p < .0001$, $^{##}p < .01$ vs. SD-SIR-SAL; $^{\wedge\wedge}p < .01$ vs. SD-SIR-OLZ; $^{\circ\circ\circ}p < .0001$ vs. FSL-SIR-SAL. $^{kk}d$ = 0.8 vs. SD-SAL; $^{mm}d$ = 1.4 vs. SD-SIR-SAL; $^{qq}d$ = 2.0 vs. FSL-SIR-SAL. **(E)** DA. $^{uu}d$ = 0.8 vs. SD-SAL; $^{tt}d$ = 1.2 vs. SD-SIR-SAL; $^{rr}d$ = 0.9, $^{ss}d$ = 1.1 vs. FSL-SIR-

SAL. **(F)** DA. °$p$ < .05 vs. SD-SAL; $^{ww}d$ = 1.2 vs. SD-SAL; $^{vv}d$ = 1.3 vs. SD-SIR-SAL; $^{zz}d$ = 1.6 vs. SD-SIR-OLZ; $^{yy}d$ = 1.0 vs. FSL-SIR-SAL. For figures 5A-F, SD-SAL and FSL-SIR-SAL are re-presented from Mncube et al., (2020). Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; OLZ, olanzapine, OFC, olanzapine+fluoxetine.

### 3.6 Plasma biochemistry

*DBH* (Figure 6A). A significant main effect of strain [$F(5, 45) = 3.503$, $p = .00093$] but no main effect of treatment or strain x treatment interaction was observed. SIR of SD- SAL decreased DBH levels versus SD-SAL controls, however, combining FSL and SIR evoked a more severe response, the latter showing some reversal by OFC but not OLZ. There was a significant reduction in DBH in SAL-treated SD-SIR compared to SD-SAL rats ($p = .0150$). Cohen's *d* showed a very large effect size reduction in DBH in SD-SIR-OLZ ($d = 1.4$) and a large effect size reduction in FSL-SIR-SAL ($d = 1.1$), and FSL-SIR-OFC ($d = 0.9$) compared to SD-SAL rats. A huge effect size decrease in SD-SIR-OLZ ($d = 2.4$) and very large effect size decrease FSL-SIR-SAL ($d = 1.8$), and a large effect size reduction in FSL-SIR-OFC ($d = 1.2$) rats was evident compared to SD-SIR-SAL rats.

*CORT* (Figure 6B). No strain x treatment interaction and no main effects of strain or treatment were evident. SIR of SD and FSL rats increased CORT levels versus SD-SAL rats, however, this increase was a smaller in FSL rats. Cohen's *d* revealed large effect size elevations of plasma CORT in SD-SIR-SAL ($d = 1.1$), FSL-SIR-SAL ($d = 0.8$), and FSL-SIR-OFC ($d = 0.9$) rats compared to SD-SAL rats. A large effect size reduction in plasma CORT was observed in OLZ-treated SD-SIR ($d = 0.8$) rats compared to SAL-treated SD-SIR animals.

*IL-6* (Figure 6C). No strain x treatment interaction and no main effects of strain or treatment were indicated. Neither SIR of SD nor of FSL rats markedly affected IL-6 levels. Cohen's *d* analysis which showed a very large effect size reduction in plasma IL-6 levels in OLZ-treated FSL-SIR rats compared to SD-SAL rats ($d = 1.2$) and a very large effect size increase compared to OFC-treated FSL-SIR ($d = 1.3$) rats. A large effect size reduction of plasma IL-6 was measured in FSL-SIR rats treated with OLZ and OFC (both $d = 1.1$) compared to SD-SIR-SAL animals.

*TNF-α* (Figure 6D). No strain x treatment interaction and no main effects of strain or treatment were evident. Neither SIR of SD nor of FSL rats markedly affected TNF-α levels. Large effect size

reductions of TNF-α levels in OLZ-treated (*d* = 1.1) and OFC-treated (*d* = 0.9) FSL-SIR rats compared to SD-SAL rats. OLZ-treated FSL-SIR rats presented with reduced plasma TNF-α compared to SD-SIR-SAL (*d* = 1.0) and SD-SIR-OLZ (*d* = 1.1). A very large effect size elevation was observed in FSL-SIR-OFC compared to FSL-SIR-OLZ (*d* = 1.3). A large effect size reduction was measured in the FSL-SIR-OFC animals compared to SD-SIR-SAL (*d* = 0.8) and compared to SD-SIR-OLZ (*d* = 0.9) rats.

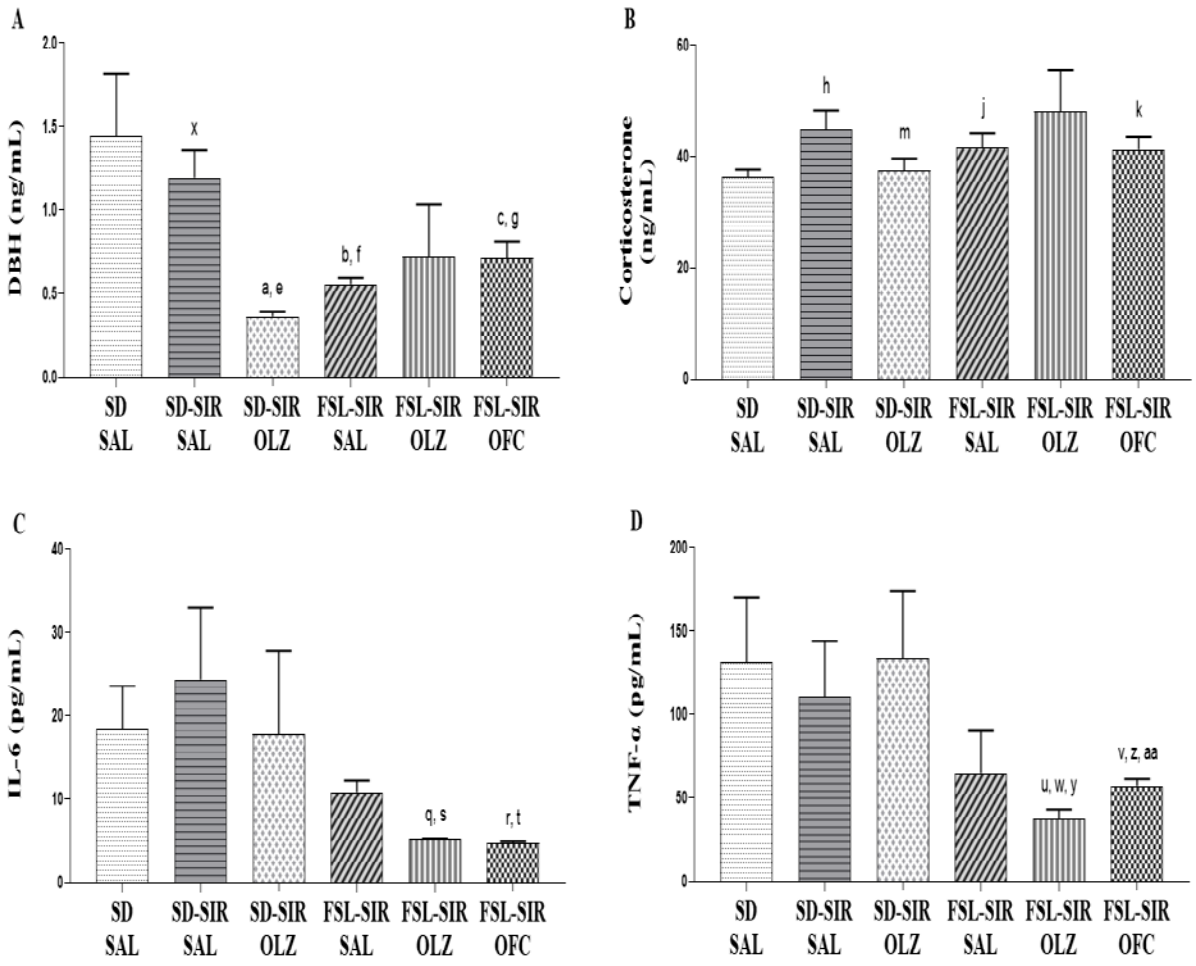


**Figure 6:** Plasma biochemistry (n = 10/group). **(A)** DBH. $^{x}p < .05$ vs. SD; $^{a}d$ = 1.4, $^{b}d$ = 1.1, $^{c}d$= 0.9 vs. SD; $^{e}d$ = 2.4, $^{f}d$ = 1.8, $^{g}d$ = 1.2 vs. SD-SIR-SAL. **(B)** CORT. $^{h}d$ = 1.1, $^{j}d$ = 0.8, $^{k}d$ = 0.9 vs. SD-SAL; $^{m}d$ = 0.8 vs. SD-SIR-SAL. **(C)** IL-6. $^{q}d$ = 1.2 vs. SD; $^{s}d$ = 1.1, $^{t}d$ = 1.1 vs. SD-SIR-SAL; $^{r}d$ = 1.3 vs. FSL-SIR-OLZ. **(D)** TNF-α. $^{u}d$ = 1.1, $^{v}d$ = 0.9 vs. SD-SAL; $^{w}d$ = 1.0, $^{z}d$ = 0.8 vs. SD-SIR-SAL; $^{y}d$ = 1.1, $^{aa}d$ = 0.9 vs. SD-SIR-OLZ; $^{z}d$ = 1.3 vs. FSL-SIR-OLZ. For figures 6A-D, SD-SAL and FSL-SIR-SAL are re-presented from Mncube et al., (2020). Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; OLZ, olanzapine, OFC, olanzapine+fluoxetine.

## 4. Discussion

Important findings from the work are that SD-SIR and FSL-SIR rats presented with significant PPI deficits that were unchanged by 5 mg/kg OLZ. OFC showed small-to-moderate improvements in PPI in FSL-SIR animals. Both SD-SIR and FSL-SIR rats displayed thigmotaxis and social impairment (increased social withdrawal and social anxiety-like behaviour), and aggressive behaviour. The latter was reversed by OFC and to some degree, by OLZ. Cortico-hippocampal NE was decreased, 5-HT increased, and DA unchanged in SD-SIR rats, while all were further depleted in FSL-SIR rats. These monoamine changes were reversed by OLZ and OFC. Elevated CORT levels were observed in SD-SIR and FSL-SIR rats. While OLZ reduced CORT in SD-SIR, CORT changes in FSL-SIR rats did not respond to OLZ or OFC. Reduced DBH was observed in the FSL-SIR and SD-SIR rats. OLZ further reduced DBH in SD-SIR but not in FSL-SIR rats, even when combined with FLX. Plasma IL-6 and TNF-α were not elevated in either FSL-SIR or SD-SIR rats, although OLZ and OFC reduced these cytokines in FSL-SIR rats.

SD-SIR and FSL-SIR rats were significantly heavier than SD-SAL rats at the end of the study, confirming the orexigenic effects of SIR in rats (Vargas *et al.*, 2016). Although OLZ is also orexigenic, reduced weight gain was observed in OLZ-treated SD-SIR rats and FSL-SIR rats, consistent with literature (Pouzet *et al.*, 2003; Shah *et al.*, 2016). Reduced weight gain following OFC-treatment was congruent with a previous study (Perrone *et al.*, 2004).

SIR increases locomotor activity in rats (Moller *et al.*, 2011) although decreased or no change in locomotor activity have also been observed (Walker *et al.*, 2019). SD-SIR and FSL-SIR rats did not exhibit hyperlocomotion with OLZ and OFC reducing locomotor activity only in FSL-SIR rats (Figure 2A). Thigmotactic behaviour indicates anxiety, a feature of schizophrenia and psychosis and has also been observed clinically in MDpsy (Ajub & Lacerda, 2018; Tandon *et al.*, 2009). Anxiety is also manifest in SIR rats (Walker *et al.*, 2019). SD-SIR and FSL-SIR rats displayed thigmotactic behaviour versus SD-SAL rats, although this was more pronounced in FSL-SIR rats (Figure 2B). While OLZ did not fully reverse thigmotaxis, it did moderately decrease thigmotaxis in SD-SIR rats ($d$ = 0.5, not shown). FLX and OFC induced a large effect size reduction in FSL-SIR rats. This is consistent with the anxiolytic properties of both OLZ and FLX (Sun *et al.*, 2010; Willner & Belzung, 2015).

In addition to anxiety, SIR is linked to deficits in social interaction or social withdrawal, aggression and hyper-reactivity to novel environments (Fone & Porkess, 2008; Forrest *et al.*, 2014; Rau *et al.*, 2015). Congruent with general rat ethology (Walker *et al.*, 2019), FSL-SIR rats exhibited less social interactive behaviour than SD-SAL controls (Figure 3A). Neither OLZ nor OFC reversed this behaviour in FSL-SIR rats (Figure 3A). SD-SIR rats did not exhibit social withdrawal. Similarly, asocial behaviour was not evident in the SD-SIR rat versus SD-SAL rats but was significant in FSL-SIR versus SD-SAL and SD-SIR-SAL rats (Figure 3B), while neither OLZ nor OFC were effective in reversing asocial behaviour in these animals (Figure 3B). Instead, OLZ-treated FSL-SIR rats exhibited worse social anxiety-like behaviour than OLZ-treated SD-SIR animals (Figure 3B), contrary to the distinct anxiolytic actions of both treatments in addressing thigmotaxis in FSL-SIR rats (Figure 3B). That FSL-SIR rats exhibited significant asocial behaviour compared to healthy controls is consistent with social anxiety seen clinically in MDpsy (Ajub & Lacerda, 2018). Despite the known anxiolytic effects of OLZ and FLX (Wąsik *et al.*, 2019; Willner & Belzung, 2015) their inability to reverse asocial behaviours suggests reduced efficacy in treating social anxiety in FSL-SIR rats (Figure 3B). Some psychosocial dysfunction may persist in MDpsy, even after depressive and psychotic symptoms have resolved with treatment, while others may persist (Tyrka *et al.*, 2006). Considering aggressive behaviours, SD-SIR rats were not significantly more aggressive than SD-SAL rats while aggression was more pronounced in FSL-SIR rats (Figure 3C). The aggressive behaviour of FSL-SIR rats is congruent with SIR literature (Jones *et al.*, 2011; Zamberletti *et al.*, 2012) as well as symptoms observed in psychosis, BD and MDpsy (Baldessarini *et al.*, 2019; Østergaard *et al.*, 2012; Scaini *et al.*, 2020). Although OLZ did not alter aggression in SD-SIR rats, it attenuated this behaviour in FSL-SIR rats (Sun *et al.*, 2010), while OFC was superior to OLZ in this regard (Figure 3C). The anti-aggressive actions of FLX (Farhan & Haleem, 2016) may explain the positive therapeutic effect when combined with OLZ in FSL-SIR rats.

SD-SIR and FSL-SIR rats presented with significant %PPI suppression compared to SD-SAL control (Figure 4), congruent with the adverse effects of early-life neurodevelopmental disturbances, i.e. SIR, on sensorimotor gating (Moller *et al.*, 2011; Veragten *et al.*, 2020). In both cohorts, OLZ treatment was ineffective in reversing said PPI suppression, albeit consistent with Bakshi *et al.* (1998). That said, OLZ at 5 mg/kg (used in the present study) was shown to dampen baseline startle magnitude (Bakshi *et al.*, 1998), which may explain its failure to reverse PPI deficits in FSL-SIR and SD-SIR rats. On the other hand, OFC caused a small effect size reversal of PPI deficits in FSL-SIR

rats versus those treated with OLZ (*d* = 0.43), possibly mediated by FLX which has been found to exert an attenuating effect on PPI deficits in a neurodevelopmental mouse model of SCZ (Yang *et al.*, 2020).

SD-SIR rats presented with reduced cortico-hippocampal NE versus SD-SAL rats (Figure 5A, B) congruent with earlier work (Trabace *et al.*, 2012). NE aberrations were normalised by OLZ treatment. FSL-SIR animals presented with a *further* diminution of cortico-hippocampal NE (Figure 5A, B) compared to SD-SAL and SD-SIR controls. This correlates with the impaired social interaction seen in the FSL-SIR rats (Figure 3A) (Swanepoel *et al.*, 2018). OLZ and OFC caused a significant rise in cortico-hippocampal NE (Figure 5A, B) consistent with literature (Zhang *et al.*, 2000). Despite this, FSL-SIR animals remained hyponoradrenergic (Figure 5A, B), supporting the lack of improvement in asocial (social anxiety-like) behaviour in OLZ- and OFC-treated FSL-SIR versus SD-SAL rats.

Elevated 5-HT was observed in SD-SIR rats but normalised by OLZ treatment. Cortico-hippocampal 5-HT levels were markedly lower in FSL-SIR rats than in SD-SAL and SD-SIR rats (Figure 5C, D), consistent with the biogenic amine theory of MD (Smolders *et al.*, 2008). These data also collate with anxiety, manifesting as less time spent in the centre of the arena (i.e. increased thigmotaxis) (Leussis & Bolivar, 2006). OLZ and OFC significantly increased 5-HT in FSL-SIR rats (Figure 5C, D), consistent with previous findings (Zhang *et al.*, 2000), which also corroborate with changes observed in locomotor activity (Figure 2A) and thigmotaxis (Figure 2B).

Cortico-hippocampal DA was similar in SD-SIR and SD-SAL rats (Figure 5E, F) congruent with literature (Trabace *et al.*, 2012; Walker *et al.*, 2019). Interestingly, OLZ had no effect on SD-SIR rat hippocampal DA but lowered cortical DA levels by a very large effect size reduction (*d* = 1.2). Depleted cortico-hippocampal DA was observed in FSL-SIR versus SD-SAL rats (Figure 5E, F), which concurs with the DA hypothesis of frontocortical hypodopaminergia in SCZ (Weinstein *et al.*, 2017) and the biogenic amine theory of MD (Villas Boas *et al.*, 2019). As described by Zhang *et al.* (2000), cortico-hippocampal DA was raised by OLZ and (moderately raised by) OFC (Figure 5E, F). This evidence for a DA deficiency in FSL-SIR rats prompts interrogation of plasma DBH levels. Plasma DBH was significantly reduced in SD-SIR rats (Figure 6A), consistent with clinical findings in SCZ (Sternberg *et al.*, 1982) (Figure 6A). FSL-SIR animals presented with an even greater reduction in

plasma DBH (Figure 6A) compared to healthy controls and SD-SIR rats. These findings provide sound support for construct validity for both a TRD and MDpsy model, where reduced plasma DBH parallels that described in MDpsy (Meyers *et al.*, 1999; Schatzberg *et al.*, 1985). Unexpectedly, OLZ reduced DBH in SD-SIR rats while no change was observed in FSL-SIR following OLZ or OFC, concordant with literature (Meyers *et al.*, 1999).

Both SCZ (Cherian *et al.*, 2019) and SIR (Mumtaz *et al.*, 2018) are associated with elevated basal plasma cortisol/CORT levels. Here SD-SIR rats demonstrated large increases in plasma CORT levels (Figure 6B) versus SD-SAL rats, reversed by OLZ treatment. Post-weaning isolation of FSL rats also raised plasma CORT correlating with findings in MDpsy that emphasise an over-stimulated HPA-axis (Heslin & Young, 2018; Schatzberg *et al.*, 1985; Tyrka *et al.*, 2006). Incidentally, a hyperactive HPA-axis is also noted in the manic phase of BD (Scaini *et al.*, 2020). OLZ or OFC however failed to abrogate increased CORT levels in FSL-SIR rats (Figure 6B). This would suggest that underlying FSL neurobiology is responsible for this difference, which may parallel the clinical differences between MDpsy and SCZ (Jeste *et al.*, 1996). Altered CORT prompts the suggestion of co-presenting immune-inflammatory changes, now considered.

TRD, SCZ and SIR are associated with elevated IL-6 and TNF-α, while BD is associated with moderate elevations in these cytokines (Brand *et al.*, 2015; Scaini *et al.*, 2020). SIR is linked to decreased IL-6 and elevated TNF-α (Brand *et al.*, 2015; Moller *et al.*, 2013). Elevated plasma IL-6 and TNF-α levels were not observed in SD-SIR rats (Figure 6C, D), consistent with other studies (Corsi-Zuelli *et al.*, 2019; Moller *et al.*, 2013), while OLZ did not alter this. Perhaps in line with a minor increase in CORT in FSL-SIR rats (Figure 6B), IL-6 or TNF-α were reduced versus SD-SAL and SD-SIR-SAL rats, albeit insignificantly. This may suggest an anti-inflammatory response aimed at protecting against combined insults (Goh *et al.*, 2020; Strauss *et al.*, 2014; Swanepoel *et al.*, 2018). OLZ and OFC treatment engendered a more profound decrease in plasma IL-6 (Figure 6C) and TNF-α (Figure 6D) in FSL-SIR versus SD-SAL and SD-SIR rats. This response is possibly mediated by increased extracellular 5-HT (Figure 5C, D) known to suppress cytokine release (Kubera *et al.*, 2005). Indeed, both FLX and OLZ have anti-inflammatory actions (Alboni *et al.*, 2016; Stapel *et al.*, 2018).

Exposing FSL rats to post-weaning SIR significantly reduces sensorimotor gating, while also inducing anxiety-like behaviour, social withdrawal, asocial and antisocial/aggressive behaviour.

Neurochemically, these animals present with reduced plasma DBH and raised CORT, congruent with MDpsy literature (Ajub & Lacerda, 2018; Fone & Porkess, 2008; Heslin & Young, 2018; Jones *et al.*, 2011; Keller *et al.*, 2007; Schatzberg *et al.*, 1985), as well as diminished cortico-hippocampal monoamines. However, plasma IL-6 and TNF-α levels remained largely unaffected. Sensorimotor gating deficits in SD-SIR and FSL-SIR rats remain refractory to OLZ or OFC, although OFC showed a large effect size reversal of this deficit in FSL-SIR rats. Neither OLZ nor OFC reversed aberrations in plasma biochemistry, and social and asocial behaviours; however, these treatments effectively reversed thigmotaxis, aggressive behaviour, and monoamine depletions relative to SAL treatment.

## 5. Limitations to the study

In an earlier study (Mncube et al., 2020, submitted), OFC failed to reverse depressive-like behaviour in FSL-SIR rats, meaning the core symptoms of psychosis and depression induced in FSL-SIR rats are refractory to OFC. While this model shows similarities to clinical MDpsy, it may more represent a model of (psychotic) BD, especially considering that OFC was not effective in reversing depressive- (Mncube et al., 2020, submitted) and psychosis-like (the present study) symptoms in FSL-SIR rats. Future work should explore this model using approved treatments for BD, such as quetiapine and lurasidone (Baldessarini *et al.*, 2019), lithium, and varying doses of OLZ in OFC.

In conclusion, exposure of FSL rats to post-weaning SIR presents with a psychotic phenotype with enhanced social impairment and aggression, together with altered MDpsy- and psychosis-related biomarkers. Moreover, these tend to be resistant to OLZ monotherapy and variably responsive to OFC treatment.

## 6. Conflict of Interest

With respect to this work, the authors declare that over the past three years, BHH has participated in advisory boards and received honoraria from Servier and Lundbeck, and has received research funding from Servier, Lundbeck, and HG&H Pharma. The authors declare that, except for income from the primary employer and research funding to BHH from the above-mentioned organizations and agencies, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services, and there are no

personal financial holdings that could be perceived as constituting a potential conflict of interest. KM and MW have no conflicts of interest to declare.

## 7. Author Contributions

KM designed the study treated the animals and collected the samples, performed behavioural and bioanalytical procedures as well as the statistical analysis, interpreted the results and prepared the first draft as well as the final version of the manuscript. MM advised on the design of the study and on the setting up of the social isolation rearing protocol and assisted with the statistical analysis. BHH devised the concept of the study, contributed to the design of the study, supervised KM, interpreted the results, co-wrote the manuscript and prepared it for submission. All the authors read and approved the final version of the manuscript for submission.

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# CHAPTER 5: CONCLUSION

## 1. General conclusion

The current study has contributed to the understanding of mood and psychotic disorders by developing a gene-x-environment based animal model of major depression with psychotic features and validating said model in terms of behaviour/symptoms, response to pharmacological intervention, and biochemistry.

MD affects an average of 269 million people worldwide and is further complicated by the increasing incidence of treatment resistance. Concurrent symptoms of psychosis with MD results in MDpsy: a more severe and difficult to treat form of depression that is understudied, underdiagnosed and, indeed, under-treated. The development of improved therapeutic strategies is hindered by the lack of animal models that effectively represent this disorder. It is the absence of such a model that has prompted the need to develop and validate a gene-x-environment model of MDpsy, as outlined in this study. This work was presented as two manuscripts that essentially address the face, construct and predictive validation of the model, and two addenda wherein additional and supportive data are presented. Here, I will now bring together this body of work into a single, unified discussion and conclusion.

Manuscript A provides a complete report of the bio-behavioural and pharmacological response of the FSL-SIR model. The FSL rat was first confirmed to exhibit a depressive-like phenotype by comparing it to SD controls. As described in that paper, FSL rats displayed increased immobility and decreased swimming behaviour in the FST; increased social withdrawal and social anxiety-like behaviour in the SIT; decreased cortico-hippocampal NE and decreased hippocampal 5-HT. In support of previous findings, depressive-like behaviours and deficits in coping behaviours as well as asocial behaviour were significantly improved by chronic FLX treatment. Cortico-hippocampal NE, social withdrawal, and climbing behaviour in the FST were not improved, consistent with a serotonergic mode of action for FLX. While frontocortical 5-HT was unchanged by treatment, hippocampal 5-HT was further and significantly reduced following FLX. Changes in regional brain 5-HT during MD and following treatment are not always predictable. FSL rats present with reduced hippocampal 5-HT transporters (SERT) compared to

healthy controls (SD rats) (Kovacevic *et al.*, 2010), which would prompt a further lowering of basal 5-HT transmission (Kovacevic *et al.*, 2010), much as described here. Furthermore, studies have shown that chronic FLX treatment reduces 5-HT production and activates post-synaptic 5-$HT_{1A}$ heteroreceptors, thereby reversing an initial increase in 5-HT-induced glutamatergic activity caused by acute SSRI treatment (Andrews *et al.*, 2015). The eventual reduction of 5-HT in limbic regions is important for therapeutic effects of antidepressants and, together with the reduced glutamatergic activity, alleviates depressive symptoms (Andrews *et al.*, 2015). Cortico-hippocampal DA, plasma DBH, CORT, IL-6, and TNF-α were unchanged in the FSL rats relative to controls which was consistent with previous findings in these rats (Carboni *et al.*, 2010; Hess *et al.*, 2009; Serova *et al.*, 1998; Tillmann *et al.*, 2018). Cortico-hippocampal DA was not altered by FLX, consistent with another animal study that associated FLX treatment with normal prefrontal-cortical DA transmission (Tanda *et al.*, 1996). DBH, IL-6, and TNF-α were not altered by FLX-treatment, consistent with clinical MD (Almeida *et al.*, 2020; Meltzer *et al.*, 1976), while CORT was significantly increased congruent with an earlier study in rats (Heydendael & Jacobson, 2010).

## 2. Validity of the FSL-SIR model for MDpsy: A critical review

This genetic animal model of MD was then exposed to 8-weeks of SIR from weaning in an attempt to exacerbate the depressive-like behaviour as well as induce treatment resistance to standard-of-care antidepressant treatment. This gene-x-environment approach was initially prompted by positive findings in an earlier dual-hit model of TRD (Brand & Harvey, 2017a, 2017b). Here, however, we based our study design on the rationale that genetically stress-sensitive individuals, when exposed to an early life neurodevelopmental challenge, will go on to develop mood *and* psychotic-like manifestations that are more severe and intractable than a genetically compromised individual alone, and certainly more than a resilient individual. As hypothesised, SIR-exposed FSL rats displayed significant immobility in the FST versus SD rats, and significant social impairment in the SIT versus FSL rats. These aberrant behaviours corroborated with significant depletions in cortico-hippocampal 5-HT and NE, respectively. Following FLX treatment, SIR-exposed FSL rats showed no improvement in depressive-like behaviour, instead exhibiting significantly *worsened* immobility and *decreased* swimming behaviour. While cortico-hippocampal NE and 5-HT were significantly improved by FLX treatment, they were not normalised and thus possibly implicated in the persistent behavioural aberrations noted above. Significantly decreased plasma DBH was observed in this model, coherent with significantly depleted cortico-hippocampal DA levels. While central

hyperdopaminergia was expected, the hypodopaminergia observed in the frontal cortex is associated with especially negative psychotic symptoms (Moller *et al.*, 2015). FLX treatment improved DA by a large effect size but did not normalise levels and left plasma DBH levels unchanged. We believe the upward trend in baseline CORT likely contributed to the inefficacy of FLX in this model; additionally, depletion of 5-HT was previously shown to prevent the antidepressant-like effects of FLX in the FST (Dailly *et al.*, 2004). Clinical studies confirm hypercortisolaemia and decreased serum DBH in TRD (Caraci *et al.*, 2018) and MDpsy (Schatzberg *et al.*, 1985) patients, supported by our findings and re-affirming our rationale for measuring these markers in plasma. Furthermore, these aberrations are associated with resistance to SSRI treatment (Samuels *et al.*, 2011; Willner & Belzung, 2015). Plasma IL-6 and TNF-α were unaltered in the SIR-exposed FSL rats, suggestive of a biological mechanism protecting against severe combined insults as in this model. This is not an unusual occurrence in pre-clinical translational models, for instance, a recent study where gestational immune activation plus SIR from weaning protected against severe pathological change (Goh *et al.*, 2020). Of the plasma biochemistry markers assayed, only TNF-α showed a large effect size decrease by FLX treatment in FSL-SIR rats. The downward trend seen here correlates with the known anti-inflammatory effects of FLX (Roumestan *et al.*, 2007). These findings provide preliminary face, construct, and predictive validity for the TRD model, supporting the hypothesis that combining a genetic animal model of MD with an early life neurodevelopmental paradigm holds promise as a suitable animal model of TRD.

TRD has therefore been observed in the SIR-exposed FSL model; however, there is still a need to elaborate on the possible presence of concurrent psychotic symptoms in the model, in particular sensorimotor gating deficits, increased anxiety, and aggression. Indeed, Manuscript A showed diminished plasma DBH and frontocortical hypodopaminergia – both linked to psychosis. At this point, it became plausible that this model may demonstrate psychotic-like behaviour concurrent with depressive-like behaviour, which would further explain the model's resistance to FLX. Moreover, with post-weaning SIR having been shown to induce psychotic-like symptoms in rats as an environmental paradigm of SCZ, its effects in FSL rats posed an interesting avenue of investigation.

In Manuscript B, SD rats reared in isolation post-weaning were confirmed to display significant sensorimotor gating dysfunction, evinced by suppressed prepulse inhibition. SD-SIR rats also

presented with trends towards reduced NE and raised 5-HT in the cortico-hippocampal regions, which have a causal association with psychotic behaviour (Meltzer *et al.*, 2006; Yamamoto & Hornykiewicz, 2004) consistent with SCZ (Risch, 1996). Isolated SD rats also presented with normal DA levels but significantly low plasma DBH, also noted in SCZ (Sternberg *et al.*, 1982), which speaks to an insufficient conversion of DA to NE. Raised CORT, another possible biomarker change associated with SCZ (Cherian *et al.*, 2019), was also significantly raised in SD-SIR versus SD control rats. However, IL-6 and TNF-α were normal in these rats, which, although observed in other post-weaning SIR animals (Corsi-Zuelli *et al.*, 2019; Moller *et al.*, 2013) is not consistent with SCZ (Moller *et al.*, 2015; Müller *et al.*, 2015). OLZ-treatment at 5 mg/kg did not reverse sensorimotor gating dysfunction or alter cortico-hippocampal DA in the SD-SIR rats, possibly due to startle suppression (Bakshi *et al.*, 1998) which we observed in this study. SD-SIR rats presented with large effect size reductions in CORT levels and cortico-hippocampal 5-HT, the latter supportive of reduced anxiety (thigmotactic behaviour) following OLZ treatment. There was a large effect size decrease in plasma DBH and normalisation of cortico-hippocampal NE in SD-SIR rats following OLZ treatment. While PPI deficits were not reversed by OLZ, there were large effect size reversals by OLZ of the reduced baseline cortico-hippocampal NE, raised frontocortical 5-HT, and raised CORT, all congruent with SCZ (Altamura *et al.*, 1999; Cherian *et al.*, 2019; Selvaraj *et al.*, 2014; Sternberg *et al.*, 1982). Therefore, while OLZ failed to reverse sensorimotor gating deficits, hence weak predictive evidence for a typical model of SCZ, other bio-behavioural parameters did show response to standard care of treatment, *viz*. OLZ.

If we now consider a possible MDpsy model, i.e. SIR-exposed FSL rats, these animals exhibited significantly increased anxiety; social withdrawal, social anxiety-like behaviour; and aggression in the SIT; significantly suppressed prepulse inhibition; significantly depleted cortico-hippocampal NE, and 5-HT; as well as large effect size reductions in cortico-hippocampal DA, plasma DBH, and elevated CORT. Moreover, OLZ treatment induced a large effect size decrease in anxiety and aggressive behaviour in the SIT, although had no effect on other social parameters or deficits in sensorimotor gating function evident in FSL-SIR rats. This is congruent with the lack of effect of OLZ monotherapy in treating clinical MDpsy compared to antidepressant-antipsychotic co-therapy (Flint *et al.*, 2013). Importantly, combined OLZ+FLX (OFC) was significantly more effective in reducing anxiety, inducing a large effect size reduction in aggressive behaviour as well as a small-to-moderate effect size improvement in prepulse inhibition in FSL-SIR rats, but was without effect

on social withdrawal and social anxiety. While both OLZ and OFC significantly raised cortico-hippocampal NE and 5-HT, and caused large effect size increases in cortico-hippocampal DA, OFC-induced changes to frontocortical NE and 5-HT levels, as well as hippocampal DA were closer to normal than those induced by OLZ treatment alone. Both treatments caused large effect size reductions in IL-6 and TNF-α relative only to SD and SAL-treated SD-SIR controls. Thus, OFC treatment is superior to OLZ monotherapy and speak to the synergistic effect of OLZ combined with FLX in bringing about more positive changes in the FSL-SIR rat. From this, the FSL-SIR rat shows good face validity for MDpsy while its *apparent* lack of strong construct and predictive validity may be indicative of unexpected biological responses to combined insults in the FSL rat, and a need for continued treatment for a longer period of time, respectively.

Addendum A investigated neuroplastic anomalies in the FSL-SIR rat compared to healthy controls, FSL and SD-SIR models by measuring novelty discrimination in the nORT and plasma BDNF to relate these cognitive behaviours to a known neuroplastic biomarker. As with the cytokines, CORT and DBH measured in manuscripts A and B, BDNF was measured in plasma to be more in line within the context of a clinically accessible biomarker. FSL-SIR animals were expected to display greater cognitive dysfunction than healthy and FSL controls but similar dysfunction to SD-SIR rats. No such cognitive dysfunction was noted. Since this unexpected data posed questions as to whether the nORT was the appropriate test for assessing cognitive function in this model, these data are presented as a separate addendum. Interestingly, FLX and OLZ did exert pro-cognitive benefits (indicated by large effect sizes) only in the FSL-SIR rats, whose memory was not markedly compromised to begin with. The combination of the two drugs did not have any such effect. FSL rats demonstrated a moderate effect size reduction in plasma BDNF, consistent with preclinical (Kirkedal *et al.*, 2019) and MD (Autry & Monteggia, 2012) literature. Neither SD-SIR nor FSL-SIR rats displayed any significant changes in plasma BDNF although SD-SIR rats (Uys *et al.*, 2017) and SCZ (Green *et al.*, 2011) are associated with decreases in BDNF. MDpsy (Huang *et al.*, 2017) presents with decreased BDNF. As far as we are aware, plasma BDNF has not been studied previously in post-weaning FSL rats, as in this study. That said, FSL rats exposed to SIR in adulthood do not exhibit differences from controls (Fischer *et al.*, 2012). Only OLZ treatment raised BDNF and only in the FSL-SIR model. OFC treatment improved neither performance in the nORT nor BDNF levels in the FSL-SIR rat.

# Chapter 5: Conclusion

Considering the study findings, it is now possible to critically evaluate the face, construct and predictive validity of the MDpsy model. We also incorporate possible validity for another psychotic mood disorder, *viz*., PBD, for which this model may have relevance. This is summarised in Table 1.

*Face validity:* MDpsy is diagnosed on the basis of psychotic features occurring *during* depressive episodes, while the presenting depressive manifestations are more intense than that seen in MD. Additionally, these patients suffer worse psychomotor agitation/retardation, anxiety, psychosocial difficulties, aggression, cognitive dysfunction and more suicide ideation, attempts and completions. SIR-exposed FSL rats displayed significant depressive-like behaviour, including immobility and decreased swimming in the FST and social withdrawal; however, both were similar to that in FSL rats. SIR-exposed FSL rats did not exhibit cognitive dysfunction but did display psychomotor agitation relative to FSL rats. FSL-SIR rats also exhibited significant psychotic-like features, including suppressed prepulse inhibition comparable to SD-SIR rats, greater anxiety, worse social withdrawal, social-anxiety-like (all significant), and aggressive behaviour (large effect size) relative to SD-SIR and healthy controls. Taken together, the FSL-SIR model does demonstrate very strong face validity for MDpsy (see Table 1).

*Construct validity:* MDpsy is characterised by hyperdopaminergia, HPA-axis hyperactivity with elevated plasma CORT, and reduced DBH levels. Other biochemical features of MDpsy include raised central NE and 5-HT. Trends toward an elevation in CORT levels and a reduction in DBH were observed in FSL-SIR rats (Manuscript B). However, no changes in plasma IL-6 and TNF-α were evident in the model (Manuscript A). Nevertheless, significant monoaminergic (DA, NE, 5-HT) depletions were observed in FSL-SIR rats, the opposite to monoaminergic findings noted in clinical MDpsy.

Concerning plasma IL-6 and TNF-α, these were investigated based on extrapolations from MD, TRD, BPD, and SCZ which have been *variably* linked to elevations of these cytokines (Brand *et al.*, 2015; Luo *et al.*, 2019; Luo *et al.*, 2016). Therefore that these pro-inflammatory cytokines were not elevated in the FSL-SIR model does not necessarily take away from the validity of the model as the roles of these cytokines in MDpsy have yet to be confirmed. FSL rats typically present with elevated NE, 5-HT, DA in the limbic system (Brand *et al.*, 2015; Overstreet *et al.*, 2005). SIR, on the other hand, is characterised by reduced cortical NE, DA, and 5-HT (Brand *et al.*, 2015; Fone & Porkess,

2008; Oehler *et al.*, 1987). This same profile is evident in the cortex of FSL-SIR rats. A similar profile was observed in the hippocampus. From this, it seems SIR exerts the more dominant effect on the monoaminergic profile of the FSL-SIR model, while the presence of two co-contributing adverse events, i.e. genetic predisposition and early life adversity, provide an explanation for the suppressed monoaminergic profile described above. The more established biological theories for MDpsy include hyperdopaminergia, hypercortisolaemia, and reduced DBH (Schatzberg *et al.* (1985), while more recent clinical data has confirmed abnormal monoaminergic activity, particularly elevated NE, 5-HT, and DA levels.

Importantly, MDpsy has been noted to present with a diagnostic switch to BD, with some patients developing BD/PBD within 2 years of diagnosis while others develop SCZ within a decade of a MDpsy diagnosis (Jääskeläinen *et al.*, 2018; Tohen *et al.*, 2012). Whether monoamine changes described in the FSL-SIR rat are truly opposite to MDpsy is debatable as monoamine status can vary according to the neuroprogressive state of the human condition (Davis *et al.*, 2014). This diagnostic instability has major implications for research, and has complicated the development of a valid translational model in animals. The current investigation is an example of this. Indeed, construct validity of a psychiatry animal model is generally the least robust attribute, mainly due to uncertainty about its causal biology (Slattery & Cryan, 2014; Söderlund & Lindskog, 2018).

Despite the above-noted limitations, we posit that the FSL-SIR rat exhibits strong construct validity for MDpsy, including hypercortisolaemia and reduced plasma DBH. However, these attributes *also* suggest construct validity for PBD. In fact, with regards to the monoamines, the variations mirrored the depressive phase of PBD (Sigitova *et al.*, 2017). Moreover, levels of DBH have been confirmed by Cubells *et al.* (2000) and Domschke (2013) to be a stable, largely genetically controlled trait in psychotic disorders, particularly in MDpsy. Schatzberg *et al.* (1985) argued that these levels do not normalise with treatment (as in the present study) making it a potential biomarker for psychotic depression. Taken together, the FSL-SIR model demonstrates weak-to-moderate construct validity for MDpsy but strong validity for PBD (see Table 1).

*Predictive validity:* OFC has been shown to be effective in treating MDpsy; proving more efficacious than either FLX or OLZ alone (Thompson *et al.*, 2019). In this study, as per clinical findings, FLX was ineffective in reversing immobility (depressive-like) in the FST while OLZ was ineffective in restoring

normal prepulse inhibition (psychosis-like) in FSL-SIR rats. As presented in Addendum B, the effect of OFC to reduce FST immobility in FSL-SIR rats was tested and in fact worsened this behaviour significantly versus SD rats. In Manuscript B, OFC was the only treatment that showed a large effect size improvement of prepulse inhibition, while in Addendum B, FLX worsened prepulse inhibition by a large effect size. While all the drug combinations significantly raised brain monoamines, OFC normalised hippocampal 5-HT and fronto-cortical DA compared to normal control. NE was raised in FSL-SIR rats following OFC but which remained significantly reduced compared to normal controls, correlating with the lack of improvement in social and asocial behaviour (Manuscript B). The effects of OFC on the monoaminergic profile of the FSL-SIR model, which varied in significance, is supportive of an antidepressant effect, although a longer treatment may have been required to see these changes translate into significant behavioural benefits in the FST (Manuscript B, Addendum B).

Together with face validity, response to chronic treatment and reversal of key behavioural (and biological) markers to treatment is the most critical attribute of an animal model (Geyer & Markou, 1995; Wang *et al.*, 2017). Considering that the efficacy of treatment is subjectively assessed by a patient based on symptoms and psychometric evaluations, that OFC-induced improvements to the FSL-SIR monoaminergic profile did not translate to improved depressive-like behaviour and did not fully reverse psychotic-like behaviour within this treatment period, weakens the predictive validity of the FSL-SIR model for MDpsy. Overall, the FSL-SIR model shows moderate-to-weak predictive validity for MDpsy (see Table 2). However, due consideration should also be given to treatment duration, where the treatment period of animals is extended, especially as clinical MDpsy has a slower response time to antidepressant treatment than MD (Rothschild, 1996).

While this model has shown *overall* strong validity for MDpsy, with some shortfalls (see Table 1), the baseline monoaminergic profile of the FSL-SIR and the behavioural response to OFC suggest potential value as a preclinical animal model of BD/PBD. Indeed, this close association with BD/PBD is evident in Table 1. Clinical studies have shown that MDpsy, in some cases, can make a diagnostic switch to BD (Busatto, 2013; Gournellis *et al.*, 2014; Heslin & Young, 2018). In fact, a family medical history of BD is a risk factor for MDpsy (Keller *et al.*, 2007). For this reason, we considered characteristics common to both (discussed in *Literature review* and *Manuscript B*). In this regard, the FSL-SIR rat may be useful as a model of PBD/BD (see validity in Table 1). The behavioural

anomalies modelled by the FSL-SIR rat, namely psychomotor agitation, depression, impaired social cognition, irritability (aggression), and psychosis (APA, 2018), are indeed congruent with those seen in clinical PBD. Additionally, the biochemical profile of this model, particularly: reduced NE, 5-HT, DA, and elevated CORT, correlates strongly with that of the depressive phase of PBD (Sigitova *et al.*, 2017; Wiste *et al.*, 2008). This connection is elaborated on further in the section on *Shortcomings and future directions*.

The data presented in Manuscripts A and B directly address the study questions and in most areas confirm the hypotheses put forward in Chapter 1, *viz.* that exposing FSL rats, a genetic model of MD, to a schizophrenia-like paradigm (SIR) will result in a gene-x-environment model of TRD that also displays psychotic-like features resistant to FLX and OLZ monotherapy. The study aims and final outcomes are now correlated and described in Table 2. However, and unexpectedly, the core behavioural deficits displayed by the model were not fully reversed by OFC. Despite this, these findings suggest that the FSL-SIR rat can be used as a model for MDpsy and possibly for PBD (see Table 1). Nevertheless, further work is necessary to provide further insight into the pathophysiology of the disorders, this in order to provide a platform to test novel treatment strategies, and to reveal novel diagnostic markers.

**Table 1:** Summary of face, construct and predictive validity of the FSL-SIR model with respect to psychotic depression (MDpsy) and psychotic bipolar disorder (PBD).

| | Criteria | FSL-SIR | Congruent for MDpsy | FSL-SIR | Congruent for PBD |
|---|---|---|---|---|---|
| Face validity | Psychomotor agitation | | ♠ ♦ | | ♠ ♦ |
| | Anxiety | | ♠ ♦ | | ♠ ♦ |
| | Cognitive impairment | | ♠ ■ | | ♠ ■ |
| | Depression | | ♠ ♦ | | ♠ ♦ |
| | Social withdrawal | | ♠ ♦ | | ♠ ♦ |
| | Social anxiety | | ♠ ♦ | | ♠ ♦ |
| | Aggression | | ♠ ♦ | | ♠ ♦ |
| | Psychosis | | ♠ ♦ | | ♠ ♦ |
| Construct validity | NE | | Elevated ■ | | Low in MDE ▲ |
| | 5-HT | | Elevated ■ | | Low in MDE ▲ |
| | DA | | Elevated ■ | | Low in MDE ▲ |
| | DBH | | Low ♦ | | Elevated ■ |
| | CORT | | Elevated ♦ | | Elevated ♦ |
| | IL-6 | Unchanged | *NCE* | | Elevated ■ |
| | TNF-α | Unchanged | *NCE* | | Elevated ■ |
| | BDNF | | Low ■ | | Low ■ |
| Predictive validity | FLX (depression) | | Poor response ♦ | | Exacerbate PBD ♦ |
| | OLZ (psychosis) | | Poor response ♦ | | *NCE* |
| | OFC (depression / psychosis) | | Responsive ■ | | Sometimes ♦ |

**Key:** ♠ – present in the clinical disorder; ♦ – Congruent with the disorder; ■ - Incongruent with the disorder; ■ – poor/weak validity; ■ – moderate validity; ■ – high/strong validity; *NCE* - no reference clinical data. Indications of significance/trends, references, comparators are detailed in the text of Chapters 3-5 and Addenda A and B.

**Table 2:** Summary of the study questions and how these were addressed by the key findings of the study.

| Source | Research Question | Summary of Findings | Experimental results | | | |
|---|---|---|---|---|---|---|
| (1.1) Manuscript A | Will FSL rats demonstrate depressive-like behaviour and altered monoamine profile and plasma biochemistry compared to SD rats? | FSL rats demonstrate bio-behavioural changes akin to depression. | ↓↓ distance<br>↑↑↑ immobility<br>↓↓↓ swimming<br>↔ climbing | ↓↓↓ social<br>↑↑↑ asocial<br>↓↓↓ FC NE<br>↓↓↓ HC NE | ↔ FC 5-HT<br>↓↓↓ HC 5-HT<br>↔ FC DA<br>↔ HC DA | ↔ DBH<br>↔ CORT<br>↔ IL-6<br>↔ TNF-α |
| (1.2) Manuscript A | Will exposing the FSL rat SIR result in a model resembling the bio-behavioural changes observed in clinical TRD? | FSL-SIR rats demonstrated a depressive-like phenotype with TRD-related biochemical changes. | ↓ distance<br>↑↑↑ immobility<br>↑↑↑ swimming<br>↑↑ climbing | ↓↓↓ social<br>↑↑↑ asocial<br>↓↓↓ FC NE<br>↓↓↓ HC NE | ↓↓↓ FC 5-HT<br>↓↓↓ FC 5-HT<br>↓ FC DA<br>↓↓ HC DA | ↓↓↓ DBH<br>↑ CORT<br>↔ IL-6<br>↔ TNF-α |
| (1.3) Manuscript A | Will the resultant animal model (FSL-SIR) demonstrate non-response to chronic treatment with FLX, as observed in clinical TRD? | FSL-SIR rats demonstrated depressive-like behaviour which was exacerbated by FLX. | ↓↓ distance<br>↔ immobility<br>↔ swimming<br>↔ climbing | ↔ social<br>↔ asocial<br>↑↑↑ FC NE<br>↑↑↑ HC NE | ↑↑↑ FC 5-HT<br>↑↑↑ HC 5-HT<br>↑↑ FC DA<br>↑ HC DA | ↔ DBH<br>↔ CORT<br>↔ IL-6<br>↔ TNF-α |
| (2.1) Manuscript B | Will SD-SIR rats demonstrate psychotic-like behaviour and altered monoamine profile and plasma biochemistry relative to SD rats? | SD-SIR rats displayed psychotic-like behaviour and an altered monoamine profile (NE, 5-HT) and plasma biochemistry (DBH, CORT) compared to SD rats. | ↔ distance<br>↑ thigmotaxis<br>↔ social<br>↔ asocial | ↔ aggression<br>↓↓↓ PPI<br>↓ FC NE<br>↓ HC NE | ↑ FC 5-HT<br>↑ HC 5-HT<br>↔ FC DA<br>↔ HC DA | ↓↓↓ DBH<br>↑ CORT<br>↔ IL-6<br>↔ TNF-α |
| (2.2) Manuscript B | Will the FSL-SIR rat exhibit behavioural and biochemical changes similar to those observed in clinical MDpsy? | FSL-SIR rats exhibited behaviour and plasma biochemistry similar to MDpsy. | ↔ distance<br>↑↑↑ thigmotaxis<br>↓↓↓ social<br>↑↑↑ asocial | ↑ aggression<br>↓↓↓ PPI<br>↓↓↓ FC NE<br>↓↓↓ HC NE | ↓↓↓ FC 5-HT<br>↓↓↓ HC 5-HT<br>↓ FC DA<br>↓↓ HC DA | ↓ DBH<br>↑ CORT<br>↔ IL-6<br>↔ TNF-α |

| | | | | | | |
|---|---|---|---|---|---|---|
| **(2.3) Manuscript B** | Will the FSL-SIR rat exhibit bio-behavioural response to chronic treatment with OLZ, as observed in clinical MDpsy? | OLZ raised depleted monoamines in FSL-SIR rats and improved anxiety and aggression but did not improve PPI deficits. | ↔ distance<br>↓↓ thigmotaxis<br>↔ social<br>↔asocial | ↓ aggression<br>↔ PPI<br>↑↑↑ FC NE<br>↑↑↑ HC NE | ↑↑↑ FC 5-HT<br>↑↑ HC 5-HT<br>↑ FC DA<br>↑ HC DA | ↔ DBH<br>↔ CORT<br>↔ IL-6<br>↔ TNF-α |
| **(2.4) Manuscript B** | Will combination therapy, that is, FLX plus OLZ result in improved bio-behavioural responses compared to either drug used alone? | FLX plus OLZ was more effective at improving bio-behavioural aberrations in the FSL-SIR compared to FLX and OLZ. | ↓↓↓ distance<br>↓↓↓ thigmotaxis<br>↔ social<br>↔ asocial | ↓ aggression<br>↑↑ PPI<br>↑↑↑ FC NE<br>↑↑↑ HC NE | ↑↑↑ FC 5-HT<br>↑↑↑ HC 5-HT<br>↑ FC DA<br>↑↑↑ HC DA | ↔ DBH<br>↔ CORT<br>↔ IL-6<br>↔ TNF-α |
| **(1.2, 1.3, 2.2, 2.3) Addendum A** | Will the FSL-SIR rat exhibit worse cognitive dysfunction vs. FSL and SD-SIR rats?<br>Will the FSL-SIR rat's cognitive performance be altered by FLX, OLZ, or OFC treatment? | FSL-SIR did not exhibit cognitive dysfunction vs. FSL, or SD-SIR rats.<br>FLX and OLZ enhanced cognitive function in the FSL-SIR while OFC made no improvements. | ↔ cognitive function vs. FSL and SD-SIR rats<br>↑ cognitive function following FLX, OLZ<br>↔ cognitive function following OFC | | | |
| **(1.2, 1.3, 2.2, 2.3) Addendum A** | How will BDNF plasma levels in the FSL-SIR rat compare to those of FSL and SD-SIR rats?<br>Will BDNF plasma concentration in the FSL-SIR rat be altered by FLX, OLZ or OFC? | FSL-SIR rat BDNF did not differ from FSL and SD-SIR rats.<br><br>FLX reduced BDNF while OLZ increased it. OFC did not alter BDNF. | ↔ BDNF vs. FSL and SD-SIR rats<br>↓ BDNF following FLX, OLZ<br>↔ BDNF following OFC | | | |
| **(1.2, 1.3, 2.2, 2.3, 2.4) Addendum B** | Will the FSL-SIR model exhibit depressive-like behaviour and will it be responsive to either OLZ or OFC treatment?<br>Will the FSL-SIR model exhibit psychotic-like behaviour and will it be responsive to FLX treatment? | OLZ and OFC exacerbated depressive-like behaviour in the FSL-SIR.<br><br>FLX exacerbated the PPI deficits occurring in the FSL-SIR. | ↑↑↑ immobility (FSL-SIR-SAL)<br>↔ immobility following OLZ<br>↔ immobility following OFC<br>↓↓↓ PPI (FSL-SIR-SAL)<br>↓↓↓ PPI following FLX (vs. SD) | | | |

**Key:** ↔ no change; ↑/↓ large effect size increase/decrease; ↑↑/↓↓ very large effect size increase/decrease; ↑↑↑/↓↓↓ significant increase/decrease

### 3. Shortcomings and future directions

The FSL-SIR preparation has shown varied validity as a model for MDpsy, with predictive validity less impressive, as described in Table 1 and Table 2. On the other hand, this "unpredictability" in as far as response to treatment is concerned especially highlights the real-life complications experienced regarding successful treatment in the clinical situation. Consequently, in order to improve its predictive validity, the model's response to treatment should be further studied.

Both core symptoms of MDpsy i.e. depression and psychosis, were manifested in this model but were not reversed by OFC treatment. That said, despite the recognised efficacy of OFC in MDpsy, it is not always effective (Rothschild *et al.*, 2004). In fact, inadequate treatment contributes to the challenge of treatment resistance (Zajecka, 2003) and here this model has demonstrated similar issues that need consideration. Future studies should therefore consider dose and duration of treatment. At the end of this study, we realised a partial response to treatment, but where treatment duration may have been too short to see a full reversal of symptoms. This is a problem even in practice where MDpsy responds slower to antidepressant therapy than MD (Rothschild, 1996). Extending treatment duration and increasing dosages may result in improvement of the measured behavioural and biochemical parameters. In addition to this, the evaluation of other drugs approved by the FDA for the treatment of BD and PBD *viz.* quetiapine, lithium, and lurasidone, should also be evaluated. In this regard, the 5-$HT_7$ receptor appears closely involved in the pathophysiology of depression where it mediates an interaction between the serotonergic system and the HPA-axis (Yohn *et al.*, 2017). Transgenic mouse studies have shown that knock-out of the 5-$HT_7$ receptor is associated with antidepressant-like effects, while 5-$HT_7$ antagonism results in rapid response to antidepressant therapy in rats (Yohn *et al.*, 2017). In fact, the augmentation effects of quetiapine (Wang *et al.*, 2016) and OLZ (Wang *et al.*, 2016) have been linked to antagonism of the 5-$HT_7$ receptor. Finally, the therapeutic benefits of lithium in treating BD but also MDpsy (Gournellis *et al.*, 2014), especially as part of an augmentation strategy, should also be considered.

No gender differences were observed in the incidence and prevalence of MDpsy (Bogren *et al.*, 2018). Female rats were therefore excluded from the study. However, the oestrous cycle poses significant challenges when using female rats in developing translational models of psychiatric

disorders. Indeed, modulatory effects of the oestrus cycle are evident in behavioural symptoms that vary between males and females and between females depending on their ovulatory stage (Kokras *et al.*, 2019; Slattery & Cryan, 2014; Walker *et al.*, 2019). An earlier study noted that post-weaning SIR differentially affects the function of the noradrenergic system in males and female rats, evidenced by changes in attentional processing of novelty and stimulated noradrenaline release in the prefrontal cortex (Atmore *et al.*, 2020). Since the present study has shown that exposing FSL rats to SIR does indeed produce an animal that exhibits both depressive- and psychotic-like behaviours (prolonged FST immobility and suppressed prepulse inhibition), it may be useful to evaluate this gene-x-environment paradigm in female rats to expand the potential for developing treatment strategies and identifying diagnostic markers.

FSL-SIR rats did not demonstrate cognitive dysfunction, contrary to what we expected. The cognitive deficits observed in MDpsy are typically worse than those in MD (Reichenberg *et al.*, 2009) but similar to SCZ (Fleming *et al.*, 2004). Although a validated nORT method previously established in our laboratory was used (Mokoena *et al.*, 2015), future testing of nORT in the FSL-SIR rat, but following some minor methodological adjustments, may reveal subtle differences in cognitive performance. Thus, shortening the inter-trial period may be beneficial since cognitive deficits in these rats may be so severe that a 90 minute inter-trial period is long enough for these rats to forget both objects from the first trial. Alternatively, novelty discrimination may be better assessed within 2 minutes as opposed to over the 5 minute period presented in Addendum A. Other behavioural tests of memory, such as the Morris water maze and radial arm were considered. However, the use of negative (cold water) and positive (edible treats) reinforcement to test for spatial memory may add a component of emotion as a possible confound in the assessment of memory. A suitable alternative test could be the object location test as a stand-alone test (to assess spatial memory) or in conjunction with the nORT (to assess spatial and recognition memory) (Denninger *et al.*, 2018). Ultimately the nORT is the preferred test in this study because it doesn't use external reinforcement to test cognition (Denninger *et al.*, 2018). Moreover, it is the more appropriate to test visual recognition, which is negatively affected in MDpsy (Schatzberg *et al.*, 2000), while visual-spatial memory is intact in MDpsy patients (Nelson *et al.*, 1998).

In this study, the FSL-SIR model exhibited behavioural symptoms of MDpsy (and PBD), although changes in monoaminergic signalling more resembled PBD depression than MDpsy (Table 1). For

this reason, future work should evaluate the model, both behaviourally, pharmacologically and biochemically, with respect to BD and/or PBD considering the cyclic nature of these disorders and the monoamine levels that fluctuate during mania or depression (Sigitova *et al.*, 2017). Here, extending the study duration could be useful to determine whether or not such fluctuations are reflected in the FSL-SIR model. The current study evaluated plasma levels of DBH, CORT, IL-6, and TNF-α to variably correlate to known clinical findings of both MDpsy and PBD. Given that there is literature informing on peripheral monoamines and their metabolites in MDpsy patients, future work could analyse these rather than central monoamines to further expound on the construct validity of this model.

The way this study has been structured, i.e. depression (Manuscript A) and thereafter psychosis (Manuscript B), considers progression from an animal model of depression to one with co-presenting symptoms of depressive- and psychosis-like symptoms. Extending the study by an additional two weeks would provide the opportunity to investigate whether behaviour and monoamine levels (in particular) in the (SAL-treated) FSL-SIR model shift towards the clinical characteristics of MDpsy or whether they remain representative of PBD depression. A proposed study layout is described in Figure 5-1, where the first 76 days mirror the current study and include blood sample collection. The study would then be continued for another two weeks during which treatment continues and behavioural tests are performed again to assess effects of prolonged treatment on behaviour and biochemistry but also to assess possible changes in the untreated/SAL-treated group. On the final day of the proposed study, blood and brain samples would be collected to determine if there were any changes in the monoamines and other markers. The blood samples and behavioural data from the first and second assessment points would be compared to assess for any shifts in either to bolster or better clarify the validity of this model for MDpsy and/or PBD.

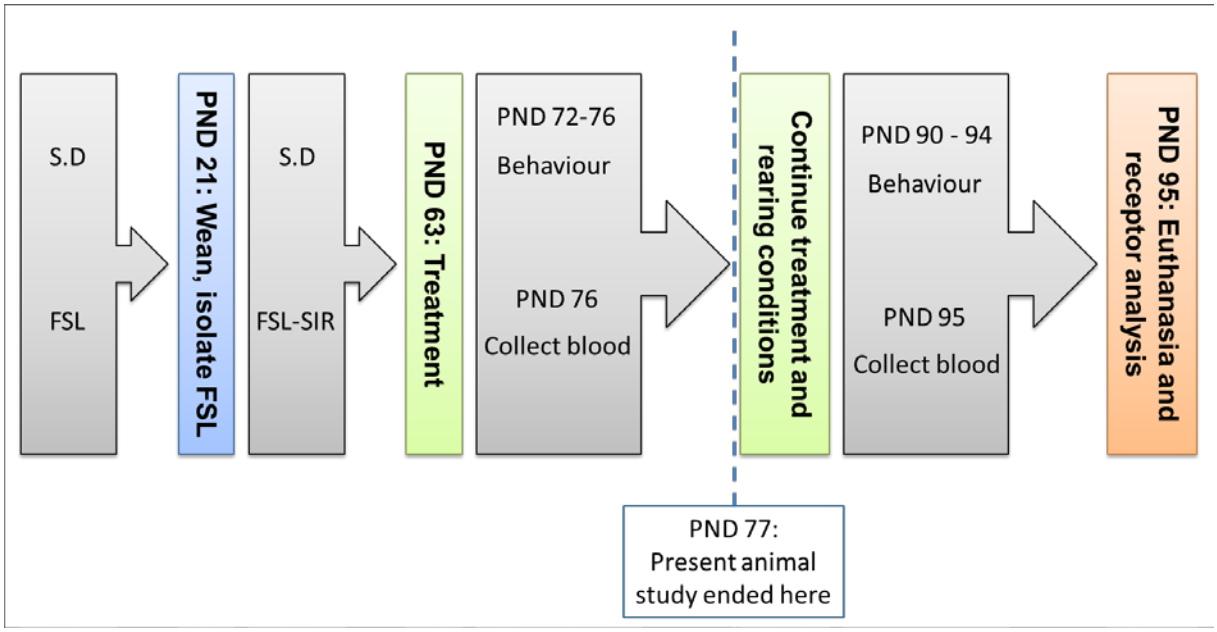


**Figure 5-1:** Proposed future study layout describing an extension of the current study. This design will test for temporal change in MDpsy and BD/PBD characteristics over time by assessing bio-behavioural manifestations two weeks apart.

Elevated glucocorticoid levels, as observed in this study, have been noted to contribute to failure of an SSRI to bolster 5-HT transmission (Samuels *et al.*, 2011). This seems to be mediated by the 5-$HT_{1A}$ receptor (Samuels *et al.*, 2011). Therefore measuring 5-$HT_{1A}$ receptor expression in the limbic structures in this model may provide an additional clue to explaining this response. Considering the 5-$HT_7$ receptor again and its purported role in TRD, cortical overexpression of this receptor has been associated with anxiety and depressive-like symptoms in adult rats (Olusakin *et al.*, 2020), symptoms also noted in FSL-SIR rats, arguing that 5-$HT_7$ receptor expression should be considered in future studies in this model.

Variation in bio-behavioural responses to pharmacological treatment is a function of dose. While this study could have benefited from a dose response analysis of FLX, OLZ and OFC, the doses used in this study were selected from a variety of pre-clinical studies for their reliability in effecting behavioural change. Due to the obligation to comply with the three R's in ethics of animal use (see the section in Chapter 1), performing this study over a range of dosages for all drug challenges would have been excessive and beyond the budget of the study. It also hinted at a possible misuse of animals. Nevertheless, now that the FSL-SIR rat has been proven to exhibit both depressive- and

psychotic-like behaviours, and also exhibited similar clinical treatment challenges (dose and duration of treatment), it would be useful to assess bio-behavioural responses as a function of drug dose and as function of time.

Another possible limitation is the presentation of this study as two separate concept manuscripts instead of combining the data into a single paper. This was done in order to best represent the study design and the long term goals of the study, *viz*. progression from an animal model presenting with symptoms of depression to a model of MDpsy. While doing this allowed certain data sets to be neatly packaged into the above set themes and hence selected for the respective papers, a single paper may have provided a more holistic interpretation of all the data; however, this would have come at the expense of brevity and simplicity. Also worth noting is that while we favoured the separation of the data across two papers, we were mindful of unnecessary wastage of animals brought on by repeating certain treatment and exposure cohorts for the two papers. This means that SD and FSL-SIR-SAL bio-behavioural datasets from Manuscript A were re-used for comparative analysis in Manuscript B. The trade-off here is that we were able to keep animal numbers to a minimum in accordance with the "refine, reduce, and replace" principle of pre-clinical research (Singh, 2012)), while at the same time allowing the reader to better relate to the data, the findings, and the study rationale.

# ADDENDUM A

**Preamble**

In **Manuscript A**, we show that exposing a genetic model of MD i.e. the FSL rat, with a social isolation rearing (SIR) paradigm of SCZ produces an animal that displays depressive-like behaviour including prolonged immobility in the FST and psychosocial impairments that are not remedied by FLX. **Manuscript B**, on the other hand, considered the psychotic-like features that this model presented, including assessment of sensorimotor gating dysfunction in the PPI test, anxiety in the OFT, and social withdrawal and anxiety as well as aggression in the SIT. However, not all available data were presented in these manuscripts. The decision to exclude certain data was made if such data risked over-complicating the manuscript, when certain data sets failed to contribute in a significant way to the final manuscript, or if the data eventually detracted from the overall impact of the paper.

Disorders of neuroplasticity, and hence memory, are common to TRD, and indeed also MDpsy, and represent an important validation criterion when developing an animal model of these conditions. Here, we present behavioural data derived from the novel object recognition test (nORT), as well as plasma brain-derived neurotrophic factor (BDNF) levels, of relevance for TRD (**Manuscript A**) and MDpsy (**Manuscript B**) originally intended for presentation in these papers. Due to these bio-behavioural markers speaking jointly towards neuroplasticity and memory, they are presented as a single manuscript.

Therefore, we describe how these bio-behavioural parameters were affected in SAL- and FLX-treated FSL-SIR rats, as depicted in Manuscript A, and SAL, OLZ and OFC treatment in SIR and FSL-SIR rats described in Manuscript B. While cognitive dysfunction is a symptom of TRD and MDpsy, these data were excluded from Chapters 3 and 4 on the grounds that these chapters sought to model the most central behavioural features of the disorder. Also, the authors considered these data would over-complicate and lessen the impact of the final presented manuscript. These data are presented in the form of a brief research report.

# Addendum A

## Fluoxetine and olanzapine improve cognition in a novel neurodevelopmental animal model of treatment resistant depression

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**Abstract**

Severe cognitive dysfunction is a characteristic feature of psychotic depression (MDpsy). We investigated cognitive function in a gene-x-environment animal model of MDpsy, its association with plasma levels of the neuroplasticity marker, brain derived neurotrophic factor (BDNF), and subsequent response to antidepressant, antipsychotic and combined treatments typically used to treat the condition. Flinder's Sensitive Line rats were reared in isolation from weaning (FSL-SIR), and subsequently treated with fluoxetine (10 mg/kg, FLX), olanzapine (5 mg/kg, OLZ), or OLZ+FLX (5 mg/kg + 10 mg/kg, OFC) for 14 days and compared to SAL-treated Sprague-Dawley (SD) rats. Cognitive function in the novel object recognition test (nORT) and plasma BDNF levels were assessed. FSL-SIR rats were not deficient in either nORT or BDNF versus SD controls. FLX decreased and OLZ increased BDNF, both with large effect sizes. FLX and OLZ significantly improved nORT in FSL-SIR versus FSL-SAL and SD-SAL rats, respectively, while OFC left BDNF and nORT unchanged. The nORT may have not been effective in demonstrating any possible cognitive dysfunction. In conclusion, these data suggest there is no correlation between plasma BDNF and memory in the MDpsy model although FLX or OLZ monotherapy increase cognition in this model.

### 1. Introduction

Major depression (MD) is a commonly occurring, often incapacitating, mental illness with great complexity in as far as presenting symptoms and causality are concerned. Treatment of MD is complicated by its heterogenous nature and co-occurring psychopathologies, including anxiety

and psychosis (Hollis & Kabbaj, 2014). When the latter is a core feature, major depression with psychotic features (or psychotic depression; MDpsy) (Bijanki *et al.*, 2014) results. MDpsy is typically treatment resistant to monotherapy with either an antidepressant or antipsychotic. However, a combination of the two in the form of a serotonin reuptake inhibitor (SSRI), fluoxetine, and an atypical antipsychotic, olanzapine, has proven to be effective in treating this disorder (Rothschild, 2013).

MDpsy is characterised by more severe MD episodes and the occurrence of psychosis manifesting as mood-incongruent or mood-congruent delusions and/or hallucinations. Moreover, the MDpsy patient suffers more intense cognitive disturbances (Keller *et al.*, 2007). Reichenberg *et al.* (2009) conducted a battery of neuropsychiatric tests to evaluate the cognitive performance of MDpsy patients versus patients with schizophrenia. They found that MDpsy patients performed poorly in verbal memory (worst), visual memory, executive function, but were equally compromised regarding attentional/speed processing (best). The similarity in cognitive dysfunction between MDpsy and SCZ patients supported earlier work by Fleming *et al.* (2004). Belanoff *et al.* (2001) found that, compared to MD patients and healthy volunteers, MDpsy patients made significantly more errors in a recognition memory test, suggesting that they had difficulty discriminating between previously presented relevant information and irrelevant new information.

Brain-derived neurotrophic factor (BDNF) is a widely expressed neurotrophin crucial in neuroplasticity as well as neuronal proliferation and survival, and is particularly relevant for mood disorders associated with cognitive dysfunction (Berk *et al.*, 2011; Brand *et al.*, 2015). Low serum levels of BDNF are observed in depression, schizophrenia, and MDpsy (Brand *et al.*, 2015). Importantly, polymorphisms of the val66met allele of BDNF were found to be associated particularly with psychotic features observed in depression (Domschke, 2013).

In manuscripts A and B (Chapter 3 and 4, respectively) we present the behavioural, biological and predictive validation of a new model for MDpsy. In **Manuscript A** we show that FSL-SIR rats exhibit prolonged immobility in the FST and poor social interaction in the SIT, while **Manuscript B** showed impaired sensorimotor gating function in the PPI, increased anxiety, poor social interaction, and aggression in the FSL-SIR rats. These behavioural traits hint at possible deficits in cognition as an additional behavioural abnormality. Indeed, plasma BDNF and memory function have been found

to co-vary in rodent models of SCZ (Faatehi *et al.*, 2019) as well as stress, and depression (Millard *et al.*, 2020). In order to better validate the model, we sought to consider cognitive dysfunction and its overlying association with BDNF with/without treatment. FSL rats perform poorly in recognition memory tasks (Oberholzer *et al.*, 2018), while SIR has also been found to decrease novelty-related behaviour in male isolates (Atmore *et al.*, 2020). We thus hypothesised that SIR-exposed FSL rats would exhibit disordered cognitive function and BDNF expression versus SD controls. To this end, we investigated behavioural performance in the novel object recognition test (NORT) in FSL-SIR rats and related this to plasma BDNF levels. Moreover, we also consider the pro-cognitive effects of fluoxetine (FLX) and olanzapine (OLZ) as monotherapies and in combination (OFC) (Gottschalk *et al.*, 2018; Marx *et al.*, 2006).

## 2. Methods

### 2.1 Animals

This study was approved by the AnimCare animal research committee (NHREC reg. no. AREC-130913-015) of the North West University (NWU) (Ethics approval number: NWU-00150-18-S5). The original colonies of FSL rats were obtained from Dr David H Overstreet, University of North Carolina, USA. All animals used were bred, supplied and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform (PCDDP) at the NWU. Male Sprague-Dawley and FSL rats were used in this study. The rats were randomly allocated to either social rearing (3 rats/cage) or social isolation rearing (1 rat/cage) at weaning on post-natal day (PND) 21 and assigned to a treatment group. The resultant cohorts were as follows: SD-SAL, FSL-SAL, FSL-FLX, SD-SIR-SAL, SD-SIR-OLZ, FSL-SIR-SAL, FSL-SIR-OLZ, and FSL-SIR-OFC. Rearing conditions were maintained for 8 weeks until PND 76 through treatment and behavioural testing protocols. All animals were exposed to the same olfactory, visual, and auditory cues, although SIR-exposed rats were deprived of social contact with peer rats during this period.

### 2.2 Drug preparation and administration

FLX (fluoxetine hydrochloride; Pubchem CID 62857; Jade Pharmaceuticals, South Africa) was first dissolved in approximately 500 µL distilled water and then made up to 10 mg/kg in physiological

saline. OLZ (olanzapine; Pubchem CID 135398745; DB Fine Chemicals (Pty) Ltd (Johannesburg, South Africa) was dissolved in approximately 200 µL 0.1 N acetic acid (Pubchem CID 176) and then in saline to make a 5 mg/kg solution. Preparation of all treatments was conducted in the Laboratory of Applied Molecular Biology (LAMB). All treatments were administered subcutaneously (s.c) according to the literature (Zhang *et al.*, 2000) as follows: 10 mg/kg FLX (Detke *et al.*, 1995), OLZ (5 mg/kg) (Heidbreder *et al.*, 2001) and OFC (10 mg/kg FLX + 5 mg/kg OLZ) for a period of 14 days. Control rats received SAL via s.c injection. Treatments were administered during the light cycle between 08:00 and 10:00 at the PCDDP.

### 2.3 nORT

The novel object recognition test (NORT) is based on the natural, explorative nature of rats when faced with novel, rather than familiar objects, and is thus used to test learning and memory. Novel object recognition deficits are noted as more time spent interacting with a familiar object rather than a novel one (Bevins & Besheer, 2006). In a rat with cognitive dysfunction, less or equal time is spent interacting with the novel and familiar object, indicating an indifference to or non-recognition of novelty. This test was performed as described by Moller *et al.* (2013) in the same arena and under the same lighting conditions as the OFT described in **Manuscript A** and **Addendum C**.

### 2.4 BDNF

Plasma BDNF was quantified using BDNF sandwich ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's protocol. Plasma as opposed to brain levels were assayed because clinical findings are mainly based on fluid sample readouts. BDNF can cross the blood brain barrier (Aldoghachi *et al.*, 2019) suggesting a parallel change in plasma and brain BDNF. This is described in **Addendum C**.

### 2.5 Statistical data analysis

Statistical analysis was performed using GraphPad Prism® 8 for Windows (GraphPad Software Inc., San Diego, CA, USA) under the supervision of the Statistical Consultation Service of the NWU. All data were checked for normality using Shapiro-Wilk's test. All data was analysed using two-way ANOVA followed by Bonferroni post hoc test. Significance was set at $p < .05$. Where no statistical

significance was indicated, Cohen's *d* was employed to indicate effect size and practical significance. Moderate ($d \geq 0.7$), large ($d \geq 0.8$) and very large ($d \geq 1.2$) effect sizes (Sawilowsky, 2009) are indicated in the figures and discussed. Data are graphically presented as mean ± SEM.

## 3. Results

Data from the different exposure and treatment cohorts presented in **Manuscripts A** and **B** are presented separately. Thus neuroplasticity-memory parameters in SAL- and FLX-treated FSL-SIR rats (**Manuscript A**) are presented in Figure A-1, with those following SAL, OLZ and OFC treatment in SIR and FSL-SIR rats (**Manuscript B**) presented in Figure A-2.

*FLX increases novelty discrimination in FSL-SIR rats tested in the nORT but reduces plasma BDNF in these rats.*

**nORT** (Figure A-1a). Two-way ANOVA demonstrated a significant main effect of rearing condition on novelty-recognition [$F(4, 44) = 2.719$, $p = .0415$]. No effect of treatment and no interaction between rearing condition and treatment were observed. Bonferroni post hoc tests showed a significant difference in novelty recognition between FSL-SAL rats and FLX-treated FSL-SIR rats, with the latter demonstrating significantly improved ability to discriminate familiar from novel ($p = .0295$). Cohen's *d* showed a medium effect size decrease in the SAL-treated FSL rat's ability to discriminate familiar from unfamiliar objects ($d = 0.7$) compared to SD-SAL rats. A moderate increase in novelty discrimination in FSL-SIR rats followed FLX treatment versus SAL-treatment ($d = 0.7$). FLX demonstrated a large effect size improvement in the discrimination index of FSL-SIR rats compared to FSL-FLX ($d = 0.8$).

**BDNF** (Figure A-1b). Two-way ANOVA did not indicate significant effects of strain, treatment, or an interaction between the two factors. Cohen's *d* analysis indicated a moderate reduction in FSL-SAL rats compared to SD-SAL rats ($d = 0.7$), and a large effect size decrease in BDNF in FSL-FLX rats ($d = 0.8$) and FSL-SIR-FLX rats ($d = 0.9$) compared to SD-SAL controls. A large effect size reduction in BDNF was evident in FLX-treated FSL and FSL-SIR rats (both $d = 1.1$) versus SAL-treated FSL-SIR rats.

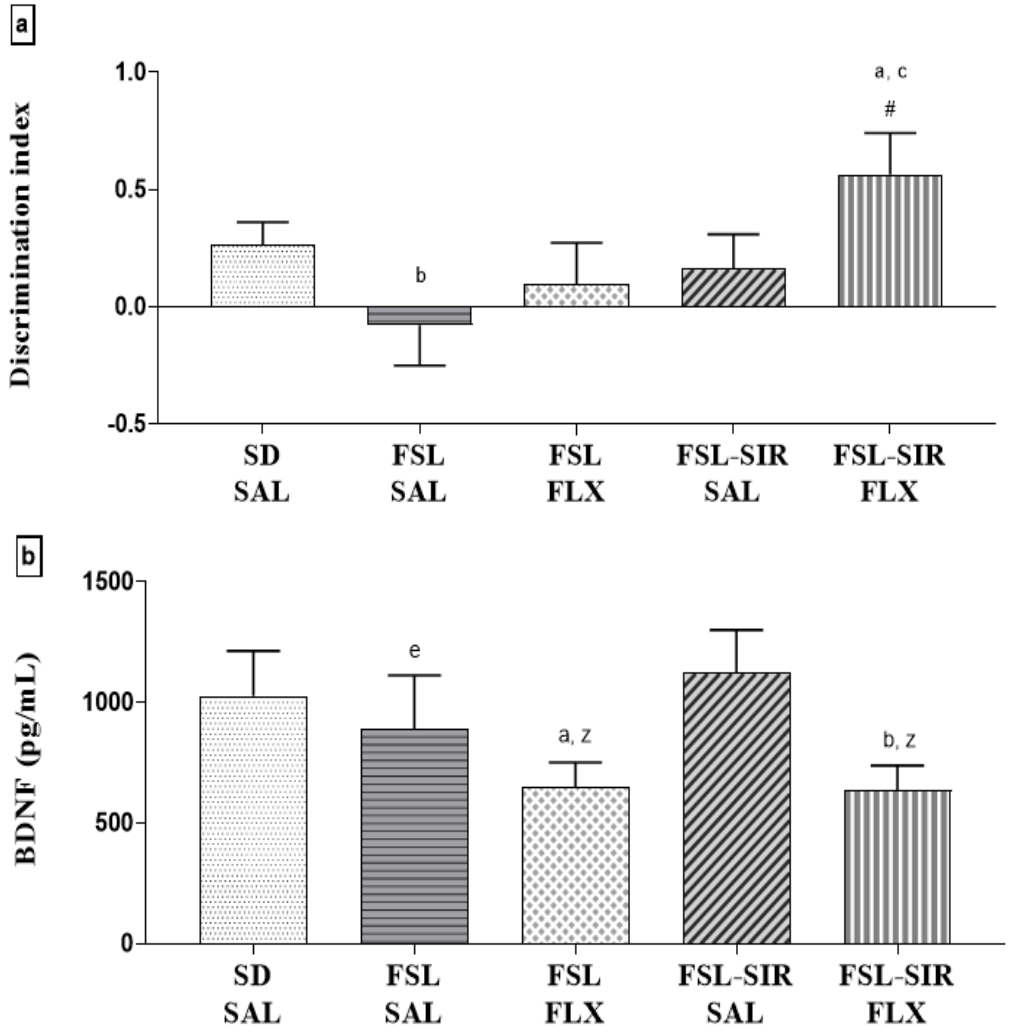


**Figure A-1:** nORT-BDNF relationship in SAL- and FLX-treated FSL-SIR rats (Manuscript A). **(a)** nORT discrimination index. $^{\#}p$ = .0295 vs. FSL-SAL; $^{b}d$ = 0.7 vs. SD-SAL; $^{a}d$ = 0.8 vs. FSL-FLX; $^{c}d$ = 0.7 vs. FSL-SIR-SAL. **(b)** BDNF. $^{a}d$ = 0.8, $^{b}d$ = 0.9, $^{e}d$ = 0.7 vs. SD-SAL; $^{z}d$ = 1.1 vs. FSL-SIR-SAL. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. BDNF, brain-derived neurotrophic factor; nORT, Novel object recognition; SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine; OLZ, olanzapine; OFC, olanzapine+fluoxetine.

*OLZ, but not OFC, causes increases in the FSL-SIR rat's ability to discriminate novel from familiar in the nORT and increases plasma BDNF in these rats*.

**nORT** (Figure A-2a). Two-way ANOVA revealed no significant effect of or interaction between rearing condition and treatment. No significant differences were revealed by Bonferroni post hoc

test. A large effect size increase in novel object recognition was observed in FSL-SIR-OLZ rats versus SD-SAL (*d* = 0.7) and versus SD-SIR-OLZ (*d* = 1.0).

**BDNF** (Figure A-2b). Two-way ANOVA did not indicate significant effects of strain, treatment, or an interaction between the two factors. Cohen's *d* analysis showed a large effect size increase in BDNF in FSL-SIR-OLZ rats versus SD-SAL, SD-SIR-SAL and FSL-SIR-OFC (all *d* = 0.9) rats and compared to FSL-SIR-SAL (*d* = 0.8).

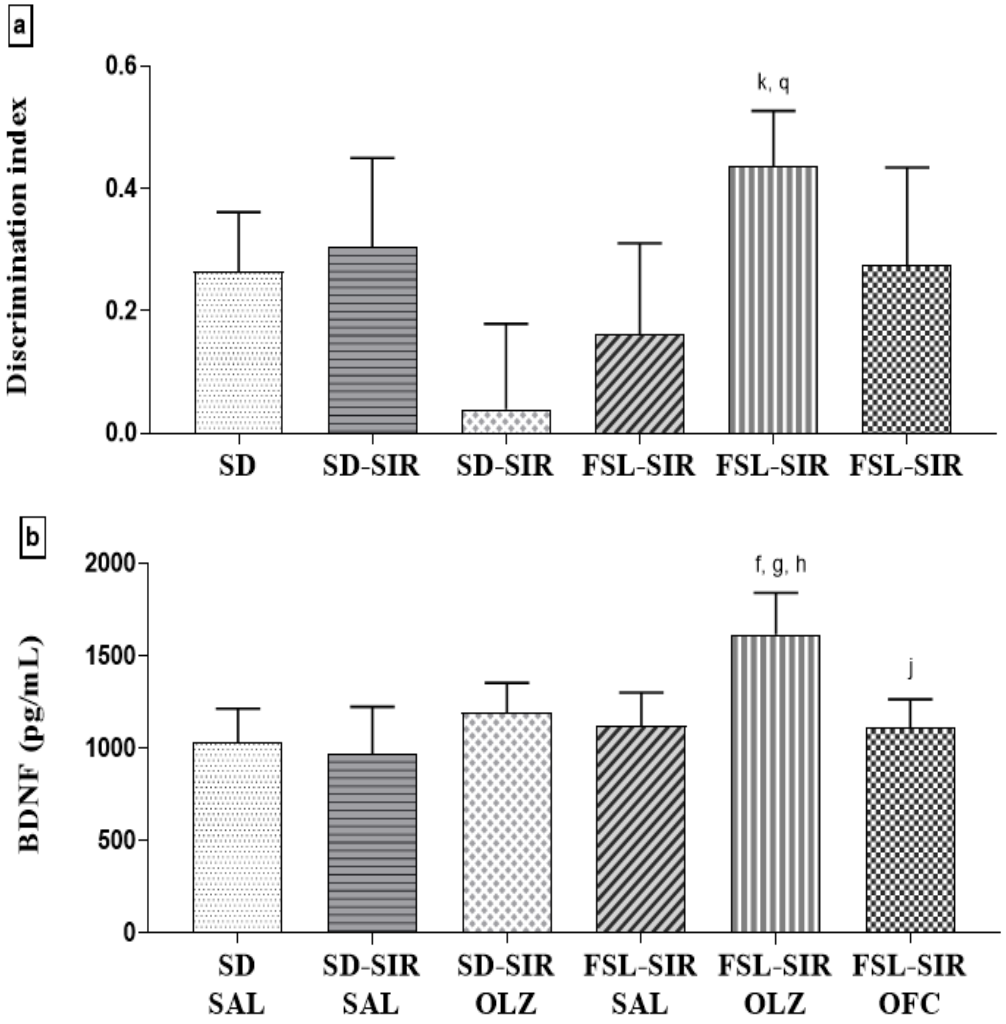


**Figure A-2:** nORT-BDNF relationship in SAL, OLZ and OFC treatment in SD-SIR and FSL-SIR rats (Manuscript B) (b). **(a)** nORT discrimination index. $^{q}d$ = 0.7 vs. SD-SAL; $^{k}d$ = 1.0 vs. SD-SIR-OLZ. **(b)** $^{f}d$ = 0.9 vs. SD-SAL; $^{g}d$ = 0.9 vs. SD-SIR-SAL; $^{h}d$ = 0.8 vs. FSL-SIR-SAL; $^{j}d$ = 0.9 vs. FSL-SIR-OLZ. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. BDNF, brain-derived neurotrophic factor; nORT, Novel object recognition; SD, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; OLZ, olanzapine; FLX, fluoxetine; OFC, olanzapine+fluoxetine.

## 4. Discussion

The purpose of this addendum (brief research report) is to describe changes in plasma BDNF and nORT in the FSL-SIR model compared to SD controls and to investigate the effects of FLX and OLZ alone and when co-administered.

While to the best of our knowledge this is the first investigation of its kind, we found that SIR-exposed FSL rats did not perform significantly worse in the nORT than FSL rats. Although unexpected, this is consistent with at least one clinical study (van Zutphen *et al.*, 2019). Likewise, BDNF levels were similar between these two groups. FSL rats showed a moderately reduced discrimination index and BDNF versus SD-SAL rats, congruent with an earlier study in FSL rats (Mokoena *et al.*, 2015) and MD literature (Douglas *et al.*, 2018; Marazziti *et al.*, 2010). FLX did not significantly alter FSL rat performance in the nORT while BDNF was decreased by a large effect size versus SD rats SAL-treated FSL rats. Concerning SAL and FLX response data in the FSL-SIR model (Figures A-1a, 1b), SAL-treated FSL-SIR rats exhibited similar discrimination index and plasma BDNF levels to SD-SAL rats. FLX decreased BDNF in FSL-SIR versus SD-SAL and FSL-SIR-SAL rats by large effect sizes but was similar to FLX-treated FSL rats. This FLX-induced decrease in BDNF in FSL-SIR rats was accompanied by a large effect size increase in novelty discrimination versus FSL-SIR-SAL and FSL-FLX rats. An inverse relationship between BDNF and nORT was noted in the FSL-SIR rats pre- and post-FLX treatment, supportive of the increased immobility observed in FLX-treated FSL-SIR rats in Manuscript A rather than a role for improved learning. This is consistent with a previous study (Borsoi *et al.*, 2015).

It has been noted that increased BDNF is correlated with improved memory (Radiske *et al.*, 2017). SIR-exposed FSL rats demonstrated similar performance in the nORT as SD-SIR-SAL rats, consistent with clinical literature (Reichenberg *et al.*, 2009). Both SD-SIR-SAL and FSL-SIR-SAL rats performed similarly to SD-SAL rats. No changes in BDNF and nORT were observed in SD-SIR rats following OLZ treatment. Concerning the OLZ and OFC response data in the FSL-SIR model (Figures A-2a, 2b), we noted a large effect size elevation in plasma BDNF which was accompanied by a large effect size increase in discrimination index versus SD-SAL and FSL-SIR-SAL, consistent with literature (Białoń *et al.*, 2020; Jena *et al.*, 2019). OFC maintained BNDF and nORT performance in the FSL-SIR rat.

# Addendum A

FSL-SIR rats did not exhibit significant cognitive deficits versus SD-SAL, FSL-SAL, and SD-SIR-SAL control rats. Both FLX and OLZ separately demonstrated pro-cognitive trends in FSL-SIR rats and hence the MDpsy model, while co-administration maintained cognitive function and BDNF levels. **Manuscripts A** and **B** showed depressive- and psychotic-like manifestations as well as social impairments in FSL-SIR rats. These behavioural changes created the expectancy of concomitant changes in memory and BDNF, which didn't occur. Although the procedure used in this study has been previously validated in our laboratory (Mokoena et al., 2015), it is plausible that another test for cognitive function other than the nORT would have delivered different results (Bryan *et al.*, 2009). Alternatively, it may warrant another explanation that is beyond the scope of this thesis. In this regard, it is also possible that the duration over which novelty recognition is assessed was too long and thus novelty could have been discriminated in the first 30 seconds of the test but the scoring of behaviour for 5 min diluted this observation since the familiar object lost its novelty and the rat moved on. It is also possible that the inter-trial period of 90 min was too long for the FSL-SIR model and that by the time the trial phase was conducted, the rats were unable to remember what they had previously explored. This in itself would suggest cognitive impairment. Thus future nORTs in this model should consider a shorter inter-trial period.

Concluding, there is a clear lack of effect on memory and BDNF levels in the MDpsy model, hence juxtaposed to changes in other bio-behavioural markers presented in Manuscripts A and B. Without a palpable explanation for this observation, and not wanting to over-complicate Manuscripts A and B with speculation, it was decided to exclude these data from the aforementioned manuscripts. Presentation in this addendum has allowed separate discussion and deliberation of these findings without allowing speculation to cloud the conclusions made in Manuscripts A and B.

# ADDENDUM B

## Flinder's Sensitive Line rats reared in social isolation exhibit depressive- and psychotic-like behaviours resistant to antipsychotic or antidepressant monotherapy

### Preamble

This Addendum reports on the effects of OLZ and OFC treatment on depressive-like behaviour in the FST and the effect of FLX on sensorimotor gating function in the PPI in FSL rats subjected to post-weaning isolation rearing (FSL-SIR), and are to be viewed against the backdrop of the manuscripts presented in Chapter 3 and 4. In **Manuscript A** we demonstrated that FSL-SIR rats exhibit depressive-like behaviour resistant to FLX treatment. This manifested as prolonged immobility in the FST that was not reversed by chronic FLX treatment (10 mg/kg x 14 days). **Manuscript A** aimed to establish SIR-exposed FSL rats as a model of treatment resistant depression (TRD), thus only antidepressant monotherapy was considered. Since this treatment resistance had been exacerbated by rearing FSL rats in isolation from weaning, a validated animal model of schizophrenia/psychosis (Heidbreder *et al.*, 2000), the underlying SIR-induced psychosis-like behaviour was hypothesised to have contributed to the lack of response to FLX treatment in the FST, as described in clinical literature (Schatzberg, 2003). Co-occurrence of depressive- and psychotic-like features is characteristic of MDpsy which is partially responsive to OLZ but most effectively treated with a combination of OLZ+FLZ, i.e. OFC (Rothschild, 2013). We therefore developed **Manuscript B** from this idea. In the latter manuscript, however, FLX monotherapy was not investigated since **Manuscript B** focused on evaluating the psychotic-like manifestations of the FSL-SIR rat and its response to OLZ and OFC treatments. Additionally, in the clinical setting, once psychotic symptoms have been noted, the patient is treated either with an antipsychotic or an antidepressant/antipsychotic combination (Rothschild, 2013) therefore FLX was not studied in **Manuscript B**. While there are no treatment guidelines that recommend antipsychotic monotherapy for MDpsy, psychotic depression in younger patients is sometimes misdiagnosed as schizophrenia (Rothschild, 1996) although this may also occur in older patients where psychotic symptoms are more prominent (Crebbin *et al.*, 2008); therefore, OLZ was included in the study described in **Manuscript B.**

# Addendum B

Here, we present behavioural data from the OFT, FST and PPI to further elaborate on the predictive validity of the FSL-SIR model. First, we report on the effect of OLZ and OFC treatments on locomotor activity (OFT) and depressive-like behaviour (FST) and then follow this up with findings from the PPI where FLX is used as treatment.

## 1. Introduction

Psychotic depression (MDpsy) is resistant to monotherapy with an antidepressant or antipsychotic (Rothschild, 1996). A combination of fluoxetine (FLX) and olanzapine (OLZ) has been shown to be most effective in treating the disorder (Luan *et al.*, 2017). In the FSL-SIR, gene-x-environment model of TRD (**Manuscript A**), FLX was ineffective at reversing depressive-like behaviour in the FST (**Manuscript A**) while OLZ did not improve on sensorimotor gating deficits in the PPI (**Manuscript B**). The combination of OLZ+FLX (OFC) improved prepulse inhibition (**Manuscript B**). In this Addendum, we investigate and discuss the effects of OLZ and OFC in FSL-SIR rat with respect to behaviour in the FST and the effects of FLX on prepulse inhibition in these same animals. For both experiments, the validated MD model (FSL), and SCZ model (SD-SIR), as well as their treatments are included. The reason for their inclusion (similar to **Manuscripts A** and **B** in **Chapters 3** and **4**, respectively) is to provide context for this data. This is done by demonstrating the resistance to the treatments by comparing the SAL- and drug-treated FSL-SIR model to a healthy control and to a "sick" control.

## 2. Methods

### 2.1 Animals

This study was approved by the AnimCare animal research committee (NHREC reg. no. AREC-130913-015) of the North West University (NWU) (Ethics approval number: NWU-00150-18-S5). The original colonies of FSL rats were obtained from Dr David H Overstreet, University of North Carolina, USA. All animals used were bred, supplied and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform (PCDDP) at the NWU. All rats were allowed free access to standard laboratory chow and water, and housed in identical transparent cages (380 mm x 380 mm x 230 mm) in an

environmentally-controlled room: constant temperature (22 ± 4°C), humidity (50 ± 20%), and a 12:12 hour light-dark cycle (lights on 06:00) with no to minimal noise. The handling of animals, as well as the experiments, adhered to the code of ethics in research, training, and testing of drugs in South-Africa, as well as to the concepts of the three R's; replacement, refinement and reduction (Singh, 2012).

### 2.2 Rearing conditions

Male Sprague-Dawley and FSL rats were used in this study. The rats were randomly allocated to either social rearing (3 rats/cage) or social isolation rearing (1 rat/cage) at weaning on post-natal day (PND) 21 and assigned to a treatment group. The resultant cohorts were as follows: SD-SAL, FSL-SAL, FSL-FLX, FSL-SIR-OLZ, and FSL-SIR-OFC (assessed in the OFT and FST as in **Manuscript A**); and SD-SAL, SD-SIR-SAL, SD-SIR-OLZ, FSL-SIR-SAL, and FSL-SIR-FLX (assessed in the PPI as in **Manuscript B**). Rearing conditions were maintained for 8 weeks until PND 76. All animals were exposed to the same olfactory, visual, and auditory cues, although SIR-exposed rats were deprived of social contact with peer rats during this period.

### 2.3 Drug preparation and administration

FLX (fluoxetine hydrochloride; Pubchem CID 62857; Jade Pharmaceuticals, South Africa) was first dissolved in approximately 500 µL distilled water and then made up to 10 mg/kg in physiological saline. OLZ (olanzapine; Pubchem CID 135398745; DB Fine Chemicals (Pty) Ltd (Johannesburg, South Africa) was dissolved in approximately 200 µL 0.1 N acetic acid (Pubchem CID 176) and then in saline to make a 5 mg/kg solution. Preparation of all treatments was conducted in the Laboratory of Applied Molecular Biology (LAMB). All treatments were administered subcutaneously (s.c) (Zhang *et al.*, 2000) according to the literature as follows: 10 mg/kg FLX (Detke *et al.*, 1995), OLZ (5 mg/kg) (Heidbreder *et al.*, 2001) and OFC (10 mg/kg FLX + 5 mg/kg OLZ) for a period of 14 days. Control rats received SAL via s.c injection. Treatments were administered during the light cycle between 08:00 and 10:00 at the PCDDP.

### 2.4 Locomotor activity in the OFT

The OFT is used to determine spontaneous locomotor activity in rodents and to control for drug-related effects on locomotion (Mokoena *et al.*, 2015). The method of Sherif and Oreland (1995) was

used. Individual rats were placed into a square arena (100 x 100 x 50 cm), facing the centre of the arena. The test was conducted in a dimly lit room illuminated with red light (40 W). The animal's behaviour was recorded for 5 min using a ceiling-mounted digital camera. The video files were analysed using Noldus Ethovision XT software (Noldus® Information Technology, Wageningen, The Netherlands) which calculated and reported the total distance (cm) travelled within the arena.

### 2.5 FST

The FST informs on depressive-like behaviour in rodents as previously described (Schoeman *et al.*, 2017). Individual rats were placed in transparent, Perspex® swim tanks containing water at ambient temperature (25°C) and allowed to swim for 7 min. FSL rats present with heightened immobility behaviour in the FST so no pre-swim was applied (Overstreet and Wegener, 2013). At the end of the 7-minute period, the rats were removed from the tanks, dried and returned to their home cages. The movements of the rats in the cylinders were digitally recorded for behavioural analysis later. These behaviours are described in Table B-1. The first and last minute of the video files were excluded from the analysis. The first minute is excluded due to enhanced buoyancy resulting from air trapped in the animal's fur when first entering the water, negating the rat's need to swim; while the last minute is excluded because the animal has reached its "maximum" immobility at this point and so inclusion of this period would dilute the effects of treatment (Oberholzer *et al.*, 2018). Immobility, swimming and climbing behaviours were scored manually by a researcher blinded to treatment and expressed in the amount of time (seconds) spent performing each behaviour.

**Table B-1:** Behaviours scored in the Forced Swim Test (FST)

| Behaviour | Description |
|---|---|
| Immobility | Passive whole-body posture where the rat makes only the most necessary movements to keep its head above water. |
| Swimming | Large forepaw movements below the surface of the water alongside movement of the body around the cylinder. |
| Climbing | Vigorous upward movement of the paws breaking the surface of the water directed against the wall of the cylinder. |

For both the OFT and FST, response to OLZ and OFC treatments were assessed in FSL-SIR compared to SAL-treated SD and FSL-SIR rats as well as to SAL- and FLX-treated FSL rats.

### 2.6 PPI

PPI is used to determine sensorimotor gating performance in humans and rodents (Shoji & Miyakawa, 2018), deficits of which correlate with clinical symptoms of disordered thoughts and distractibility (Forrest *et al.*, 2014) evident in psychosis (American Psychiatric Association, 2013). PPI was assessed in two ventilated and illuminated, sound-attenuating startle chambers (SR-LAB, San Diego Instruments, San Diego, USA), as described previously (Moller *et al.*, 2011). Acoustic startle response (ASR) represented habituation in response to repeated delivery of startling stimuli (Swanepoel *et al.*, 2018). Per cent PPI (%PPI) for each prepulse+pulse trial was calculated using the following formula: % PPI=[100−(startle response for PREPULSE+PULSE trial)/(startle response for PULSE ALONE trial)×100] (Swanepoel *et al.*, 2018). Average %PPI values across the four prepulse intensities were calculated and used.

Here, the FSL-SIR response to FLX treatment was assessed and compared to SAL-treated SD and FSL-SIR rats as well as to SAL-and OLZ-treated SD-SIR rats.

### 2.7 Statistical analysis of data

Statistical analyses were performed using GraphPad Prism® 8 for Windows (GraphPad Software Inc., San Diego, CA, USA) under the supervision of the Statistical Consultation Service of the NWU. ASR data was analysed using a repeated measures two-way ANOVA. OFT, FST, and PPI were analysed using a two-way ANOVA. In both cases, the selected ANOVA was followed by Bonferroni post-hoc test. Significance was set at $p < .05$. Where significance was not found, Cohen's *d* analysis was employed. Large effect sizes are indicated by $d \geq 0.8$ and very large effect sizes by $d \geq 1.2$ (Sawilowsky, 2009). Only large and very large effect sizes are noted in the figures and discussed. Data are graphically presented as mean ± SEM.

## 3. Results

### 3.1 OFT

*Distance travelled* (Figure B-1). Two-way ANOVA revealed a significant main effect of rearing condition [$F(5, 55) = 5.750$, $p = .0002$] but none of treatment and no interaction between the two factors. FSL-FLX ($p = .0350$) and FSL-SIR-OFC ($p = .0002$) rats travelled a significantly shorter distance versus SD-SAL rats. OFC-treated FSL-SIR rats also travelled significantly less than SAL-treated FSL-SIR rats ($p = .0033$). Cohen's *d* analysis showed a very large effect size decrease in distance travelled by SAL-treated FSL rats compared to SD-SAL rats ($d = 1.5$), while a large effect size increase in activity was observed in SAL-treated FSL-SIR rats versus FSL-SAL ($d = 0.9$) and versus FSL-FLX rats ($d = 1.1$). OLZ-treated FSL-SIR rats travelled less than SD-SAL rats with a large effect size of $d = 1.0$. OFC reduced the distance travelled by FSL-SIR rats compared to FSL-SAL rats ($d = 1.0$) and OLZ-treated FSL-SIR rats ($d = 1.0$).

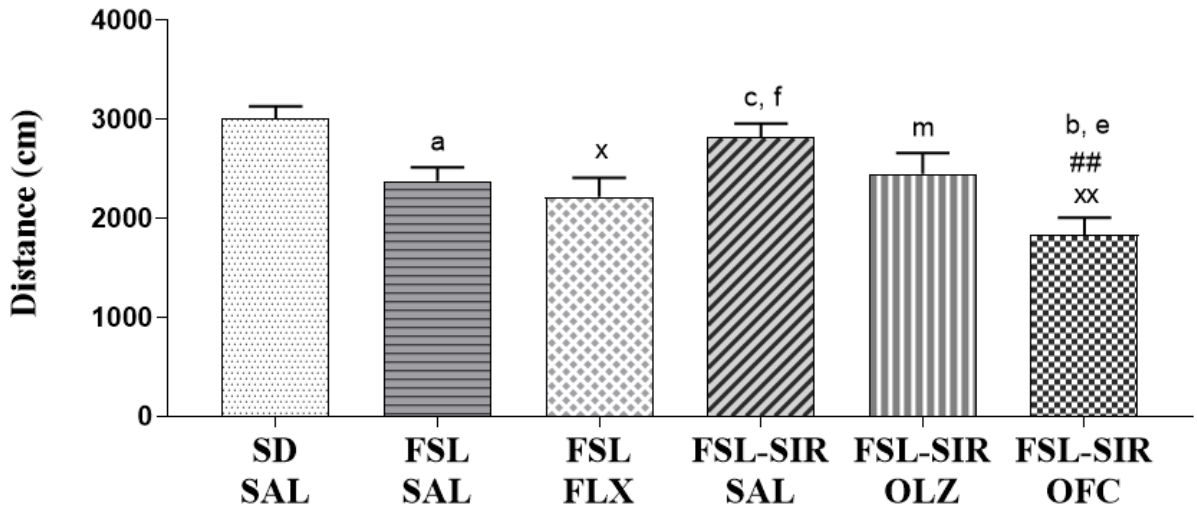


**Figure B-1:** Locomotor activity of SAL-treated SD and SAL- and drug-treated FSL and FSL-SIR rats as measured by **d**istance travelled in the OFT. $^{xx}p < .01$, $^{x}p < .05$ vs. SD-SAL; $^{\#\#}p < .01$ vs. FSL-SIR-SAL; $^{a}d = 1.5$, $^{m}d = 1.0$ vs. SD-SAL; $^{c}d = 0.9$, $^{b}d = 1.0$ vs. FSL-SAL; $^{f}d = 1.1$ vs. FSL-FLX; $^{e}d = 1.0$ vs. FSL-SIR-OLZ. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine; OLZ, olanzapine; OFC, olanzapine+fluoxetine.

### 3.2 FST

*Immobility* (Figure B-2a). Two-way ANOVA revealed a significant main effect of rearing condition [$F(5, 55) = 12.95$, $p < .0001$] but none of treatment and no interaction between the two factors. Both FSL-SAL ($p < .0001$) and FSL-SIR-SAL ($p = .0092$) rats spent significantly more time immobile than SD-SAL rats. Both OLZ and OFC treatment (both $p < .0001$) maintained significant immobility versus SD-SAL rats. FLX treatment resulted in significantly decreased immobility in FSL rats compared to FSL-SAL ($p = .0021$), FSL-SIR-OLZ ($p = .0006$), and FSL-SIR-OFC ($p = .0001$) rats. Cohen's *d* analysis showed very large effect size increase in immobile behaviour in FSL-SIR treated with OLZ ($d = 1.2$) and OFC ($d = 1.8$) versus SAL treatment.

*Swimming* (Figure B-2b). Two-way ANOVA revealed a significant main effect of rearing condition [$F(5, 55) = 20.28$, $p < .0001$] but no effect of treatment and no interaction between the two factors. SAL-treated FSL, OLZ-treated FSL-SIR and OFC-treated FSL-SIR rats (all $p < .0001$) demonstrated significantly reduced swimming behaviour compared to SD-SAL controls. Both FSL-FLX ($p < .0001$) and FSL-SIR-SAL ($p = .0044$) swam significantly more than FSL-SAL rats. OLZ- and OFC-treated FSL-SIR rats (both $p < .0001$) swam significantly less than FLX-treated FSL rats. OLZ ($p = .0025$) and OFC ($p = .0294$) significantly reduced swimming in FSL-SIR rats compared to SAL treatment. A large and a very large effect size decrease in swimming was observed in FSL-SIR-SAL rats compared to SD-SAL ($d = 1.0$) and FSL-FLX rats ($d = 1.3$), respectively.

*Climbing* (Figure B-2c). Two-way ANOVA revealed a significant main effect of rearing condition [$F(5, 55) = 2.821$, $p = .0244$] but no effect of treatment and no interaction between the two factors. A large effect size increase in climbing was observed in FSL-SAL ($d = 0.8$), FSL-FLX ($d = 1.2$), FSL-SIR-SAL ($d = 1.2$), and FSL-SIR-OLZ ($d = 0.8$) rats versus SD-SAL rats. A large effect size reduction in climbing was observed in FSL-SIR-OFC compared to FSL-FLX and FSL-SIR-SAL rats (both $d = 1.0$).

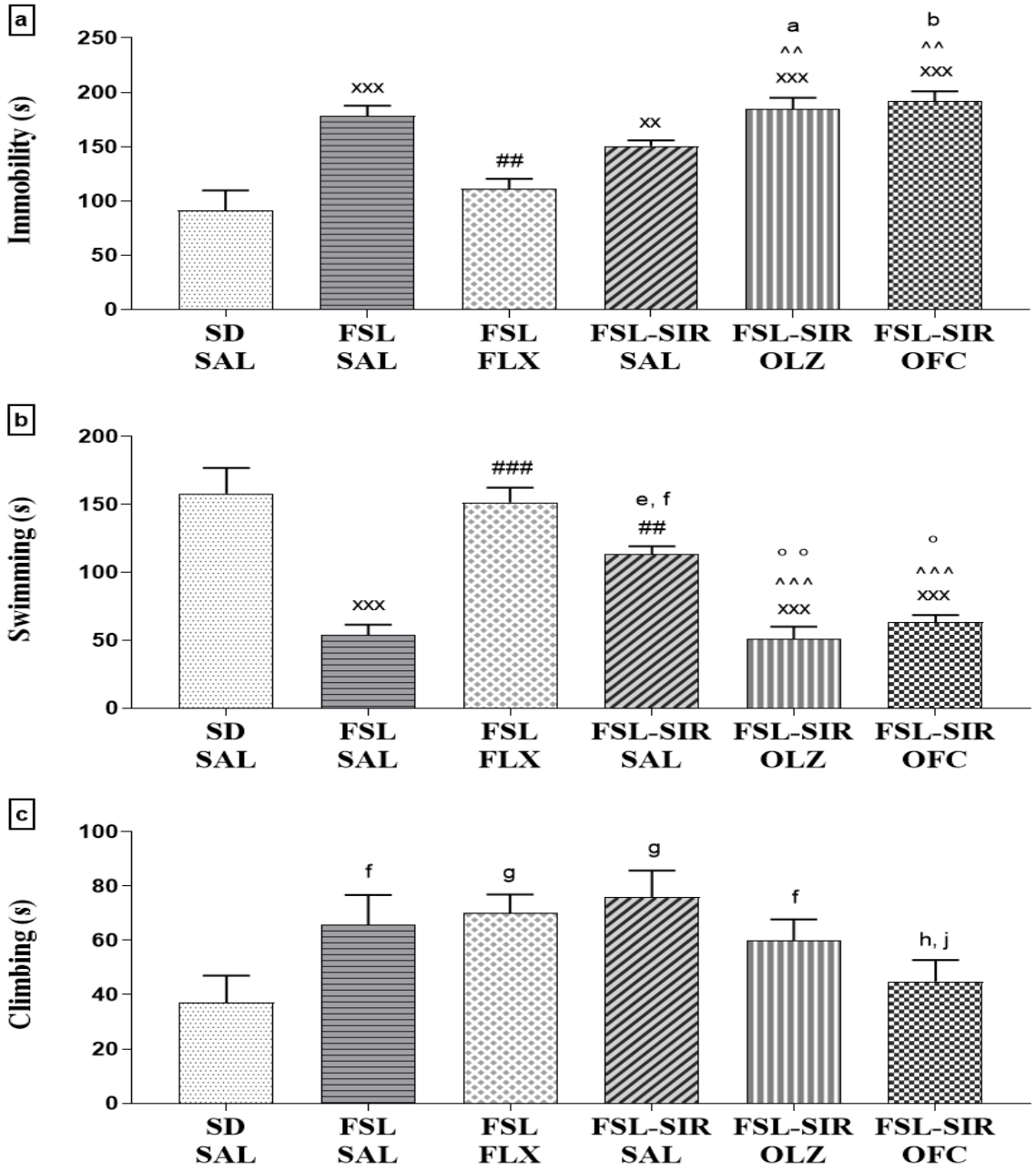


**Figure B-2:** Depressive-like behaviour in SAL-treated SD and SAL- and drug-treated FSL and FSL-SIR rats as measured in the FST. **(a)** Immobility in the FST. $^{xxx}p < .0001$, $^{xx}p < .01$ vs. SD-SAL; $^{\#\#}p < .01$ vs. FSL-SAL; $^{\wedge\wedge}p < .01$ vs. FSL-FLX; $^{a}d = 1.2$, $^{b}d = 1.8$ vs. FSL-SIR-SAL. **(b)** Swimming behaviour in the FST. $^{xxx}p < .0001$ vs. SD-SAL; $^{\#\#\#}p < .0001$, $^{\#\#}p < .01$ vs. FSL-SAL; $^{\wedge\wedge\wedge}p < .0001$ vs. FSL-FLX; $^{\circ\circ}p < .01$, $^{\circ}p < .05$ vs. FSL-SIR-SAL; $^{e}d = 1.0$ vs. SD-SAL; $^{f}d = 1.3$ vs. FSL-FLX. **(c)** Climbing behaviour in the FST. $^{f}d = 0.8$, $^{g}d = 1.2$ vs. SD-SAL; $^{h}d = 1.0$ vs. FSL-FLX; $^{j}d = 1.0$ vs. FSL-SIR-SAL. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; FLX, fluoxetine; OLZ, olanzapine; OFC, olanzapine+fluoxetine.

## 3.3 PPI test

### 3.3.1 Acoustic startle response

Repeated measures ANOVA indicated significant main effects of BLOCK [$F(3, 75) = 21.51$, $p < .0001$], treatment [$F(4, 25) = 3.097$, $p = .0336$], and strain [$F(25, 75) = 4.045$, $p < .0001$]. No interaction between factors was indicated. Startle response was highest in the first block across all cohorts; thereafter, in-session habituation was observed. Significant startle suppression was observed in BLOCK 1 in FSL-SIR-FLX rats compared to SD-SAL ($p = .0003$) and SD-SIR-OLZ ($p = .0067$). This is depicted in Figure B-3.

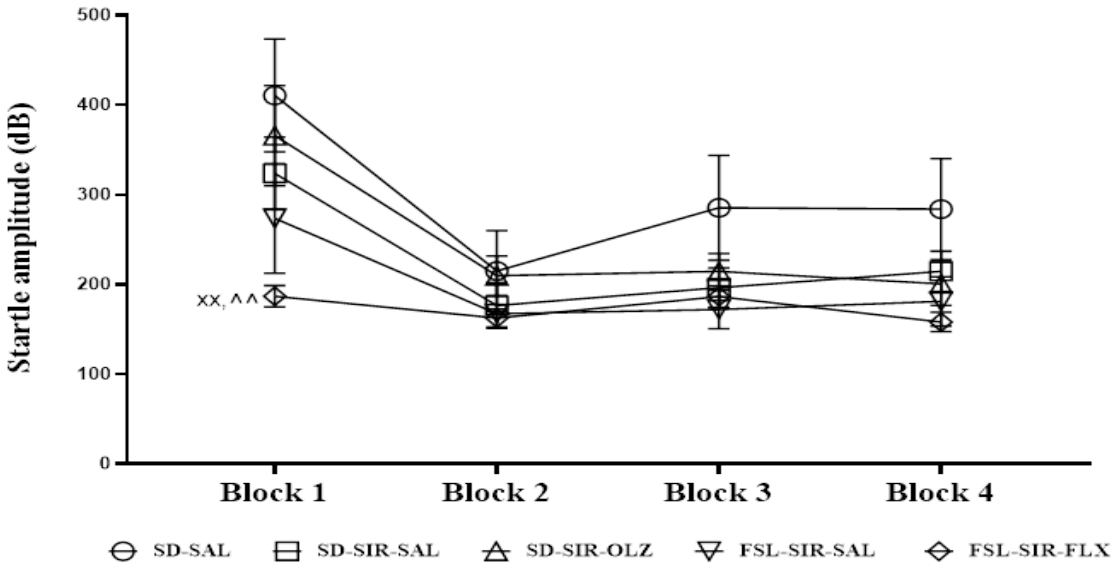


**Figure B-3:** ASR measured as startle amplitude (dB in SAL-treated SD, and SAL- and FLX-treated FSL-SIR rats.. $^{xx}p < .01$ vs. SD-SAL; $^{\wedge\wedge}p < .01$ vs. SD-SIR-OLZ (both BLOCK 1). Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; OLZ, olanzapine; FLX, fluoxetine.

### 3.3.2 PPI

Two-way ANOVA revealed a significant main effect of strain [$F(4, 44) = 6.427$, $p = .0004$] on %PPI. No effect of treatment and no strain x treatment interaction were indicated. SD-SIR-SAL ($p = .0112$), SD-SIR-OLZ ($p = .0079$), FSL-SIR-SAL ($p = .0165$), and FSL-SIR-FLX ($p = .0002$) demonstrated

significantly reduced %PPI versus SD-SAL. A moderate effect size decrease in %PPI in FSL-SIR rats followed FLX treatment versus SAL treatment (*d* = 0.7, not shown). This is shown in Figure B-4.

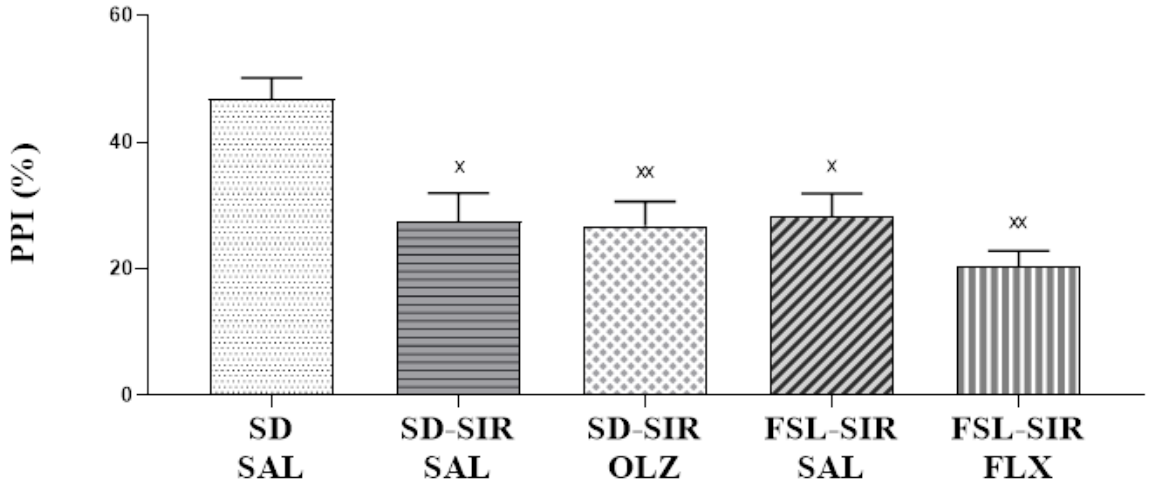


**Figure B-4:** Sensorimotor gating function as measured in the PPI test in SAL-treated SD, and SAL- and FLX-treated FSL-SIR rats. Average %PPI. $^{xx}p < .01$, $^{x}p < .05$ vs. SD-SAL. Data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test and Cohen's *d* analysis. Data are presented as mean ± SEM. SD-SAL, Sprague-Dawley; FSL, Flinders' Sensitive Line; SIR, social isolation rearing; SAL, saline; OLZ, olanzapine; FLX, fluoxetine.

## 4. Discussion

In **Manuscript A**, we showed that SIR-exposed FSL rats displayed depressive-like behaviour in the FST that was resistant to FLX treatment. In **Manuscript B**, we showed that the FSL-SIR rat displayed impaired sensorimotor gating function in the PPI that were resistant to OLZ treatment. Considering these findings and the clinical MDpsy literature (Rothschild, 1996, 2013), we hypothesised that SIR introduced psychotic-like features in FSL rats that engendered the condition resistant to treatment. We, therefore, investigated the effects of OLZ and OFC on depressive-like behaviour as investigated in **Manuscript A** to extend the predictive validity of the FSL-SIR rat as an MDpsy model. Thereafter, we investigated the efficacy of FLX in treating psychosis-like manifestations in FSL-SIR rats, i.e. sensorimotor gating dysfunction in the PPI test as in **Manuscript B** in order to parallel treatment outcomes when psychotic symptoms are missed resulting in inappropriate treatment being prescribed. Here our data describe the treatments to be ineffective in reversing the anomalous behaviours.

# Addendum B

Depressive-like behaviour

OLZ and OFC decreased locomotor activity in the FSL-SIR rat relative to SD rats. Both treatments also *worsened* depressive-like behaviour in the FST, demonstrated by increased immobility and decreased coping behaviour (swimming behaviour) compared to SD-SAL and FSL-SIR-SAL rats. OLZ increased climbing behaviour in the FSL-SIR versus SD-SAL rats while OFC decreased climbing versus FSL-SIR-SAL rats. The lack of behavioural response to FLX (Manuscript A) establishes a TRD model in rats, while worsening of depressive symptoms following OLZ treatment parallels clinical MDpsy (Rothschild, 1996). Clinically, OFC is effective in the treatment of MDpsy (Luan *et al.*, 2017) yet this was not observed in the FSL-SIR rats. It should be noted that while OFC decreased locomotor activity and climbing, these parameters were reduced to levels similar to FSL-FLX and SD-SAL rats, respectively, and so suppressed locomotor activity has not masked a beneficial antidepressant-like response in the FST. Instead, these results, considered alongside the monoaminergic profiles of the OLZ- and OFC-treated FSL-SIR rats (**Manuscript B**), suggest that OFC may indeed be effective in alleviating depressive-like behaviour. However, the neuroplastic changes that would herald an improvement in symptoms have yet to be established. This is consistent with the clinical issue of MDpsy patients receiving appropriate treatment for too-short a duration where depressive symptoms in MDpsy show a slower response to treatment (Rothschild, 1996).

Psychotic-like behaviour

SIR significantly suppressed sensorimotor gating in SAL-treated FSL rats. OLZ was not effective in reversing this deficit (**Manuscript B**) while OFC partially reversed this response. Here, FLX maintained sensorimotor gating suppression in the FSL-SIR rat and caused a further moderate reduction in PPI compared to SAL-treatment ($d$ = 0.7, not shown). **Manuscript A** showed a significant increase in cortico-hippocampal 5-HT following FLX treatment, which may offer some explanation for these findings. Indeed, FLX-induced elevations in 5-HT decrease PPI via $5\text{-HT}_{1B}$ receptor activation (Dulawa *et al.*, 2000). The $5\text{-HT}_{1B}$ receptors function as inhibitory autoreceptors located presynaptically on serotonergic neurons where they inhibit 5-HT neuronal release (du Jardin *et al.*, 2018). FSL rats have a high density of these receptors which correlates with the typically lower tissue levels of 5-HT found in these rats and in the resultant depressive-like symptoms (Nishi *et al.*, 2009).

# Addendum B

MDpsy is characterised by both psychosis and depression, however, the psychotic symptoms are often missed resulting in patients receiving inadequate treatment, i.e. antidepressant monotherapy (Rothschild, 2013). Here we determined that while OLZ and OFC exacerbated these depressive-like symptoms, OFC induced cortico-hippocampal monoaminergic changes described in Manuscript B are supportive of a positive treatment response. Furthermore, the large effect size improvement in PPI in FSL-SIR rats following OFC treatment noted in **Manuscript B** confirms a response, albeit a slow one. The worsening of psychotic-like symptoms, i.e. reduced PPI, following FLX treatment further confirmed the treatment resistance observed in FSL-SIR rats reported in **Manuscript A** and re-affirmed that underlying psychosis in MD is not responsive to FLX.

In conclusion, these additional data are useful in interpreting the overall findings described in **Manuscripts A** and **B** by showing that OFC treatment is superior to monotherapy with either FLX or OLZ with regards to psychotic-like symptoms and enhanced monoaminergic signalling. In addition, OFC eradicated aggression in the FSL-SIR rat while OLZ only reduced it (**Manuscript B**). While the depressive-like symptoms were worsened in the FSL-SIR rat following OFC treatment, the positive monoaminergic changes following OFC suggest a longer duration would improve depressive-like symptoms. This brings to light the need to extend treatment duration in the FSL-SIR model to overcome this limitation.

# ADDENDUM C: METHODS

This addendum provides detailed descriptions of the behavioural and analytical methods used in this thesis.

## 1. Behavioural tests

### 1.1 nORT

This test was used in **Addendum A** to assess cognitive function.

Rats were placed in an empty OFT arena for a 10 minute habituation period 24 hours before the nORT. On the day of the nORT, the arena was cleaned before placing two identical objects on diagonally opposite corners of the arena. The rat was returned to the arena and given 5 min to explore the arena and objects. This is the acquisition phase of the study. After this phase, the rat was returned to its homecage where it remained for a 90 min inter-trial rest period. During this time, the arena and toys were cleaned with 70% (vol/vol) ethanol to avoid olfactory trails (Leger *et al.*, 2013). For the trial phase, one of the toys from the acquisition phase is returned to the arena and a *new* toy was introduced into the arena, both placed in the same position as in the acquisition phase. The rats were then re-introduced into the arena and allowed another 5 min to explore the two objects and arena. Their behaviour were recorded and scored using Noldus Ethovision XT according to object exploration defined as sniffing, licking or touching of objects with forepaws (Mokoena *et al.*, 2015).

Objection recognition was expressed as the discrimination index calculated as follows: % DI = [(time spent exploring novel object – time spent exploring familiar object)/(total time spent exploring both objects)]. The more time spent interacting with the new, unfamiliar object, the closer the discrimination index value was to 1. Novelty recognition suggests intact or improved recognition memory (Bevins & Besheer, 2006).

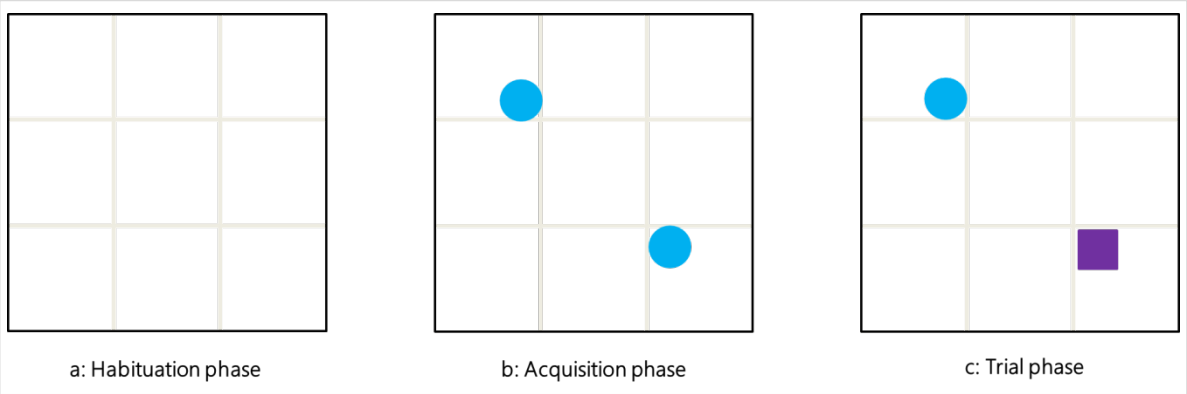


**Figure C-1:** nORT test phases. **(a)** Habituation phase. In this phase, the rat is allowed to explore an empty arena for 5 min. **(b)** Acquisition phase (24 h after the habituation phase). Two identical objects were placed in the arena for exploration for 5 min. **(c)** Trial phase (90 min after the end of the acquisition phase). One object identical to the acquisition phase and a novel object are placed in the arena for 5 min.

## 1.2 PPI

PPI was used to determine sensorimotor gating performance as described by Shoji and Miyakawa (2018), deficits of which correlate with clinical symptoms of disordered thoughts and distractibility (Forrest *et al.*, 2014) evident in psychosis (American Psychiatric Association, 2013). PPI was assessed in two ventilated and illuminated, sound-attenuating startle chambers (SR-LAB, San Diego Instruments, San Diego, USA), Each startle chamber consists of a transparent, non-restrictive Plexiglas cylinder (diameter 8.2 cm, length 20 cm) mounted atop a Plexiglas frame (the stabilimeter). A speaker mounted above the cylinder emits acoustic noise bursts. The startle response manifests as a contraction of skeletal muscles which shortens the torso elevating the back and causing a force to be directed through the feet to the floor of the cylinder (Swerdlow, 2009). This reactive flinch is detected and transduced into analogue signals by a piezoelectric accelerometer attached to the frame and digitised by SR-LAB software. The test session was made up of four segments against a 68 dB white noise background. The first (BLOCK 1) and last segment (BLOCK 4) were the first and last 10 pulse-alone stimuli each lasting 40 ms. The second and third (BLOCK 3) segments included pulse-alone, no-pulse and prepulse trials. After BLOCK 1, the test session continued with 20 pulse-alone trials of 115 dB, 40 prepulse trials (a single 115 dB pulse preceded by 20 ms long non-startling prepulse stimulus at intensities of 72, 76, 80 or 84 dB), and 10 no pulse trials during which no stimulus was presented. The last 10 trials were BLOCK 4. BLOCK 1 and BLOCK 4 as well as the 20 pulse-alone stimuli included in the PPI trials (BLOCK 2 and BLOCK 3)

were used to obtain a measure of mean startle amplitude demonstrating habituation in response to repeated delivery of startling stimuli (Swanepoel *et al.*, 2018). This is represented by acoustic startle response (ASR).

Per cent PPI (%PPI) for each prepulse+pulse trial was calculated using the following formula: % PPI=[100−(startle response for PREPULSE+PULSE trial)/(startle response for PULSE ALONE trial)×100] (Swanepoel *et al.*, 2018). Throughout the study, no significant interaction between prepulse levels and pharmacological treatment was found, the four %PPI values were therefore collapsed to represent average %PPI.

This protocol is adapted from methods described by Moller *et al.* (2011) and (Swanepoel *et al.*, 2018).

## 2. Analytical methods

### 2.1 HPLC

This section describes the method used to determine rat frontocortical and hippocampal tissue levels of NE, 5-HT, and DA using high performance liquid chromatography system (HPLC) with an electrochemical detector (ECD). The method is a standard in-house method described by Viljoen *et al.* (2018) and used for the work presented in **Manuscripts A** and **B** (**Chapters 3** and **4**, respectively).

#### 2.1.1 Instruments and materials

An Agilent 1200 series HPLC (Agilent Technologies, California, USA), equipped with an isocratic pump, auto sampler coupled to an ESA Coulchem III Electrochemical detector with a coulometric flow cell (Model 5011A High Analytical Cell and Guard cell 5020), and Chromeleon® Chromatography Management System software version 6.8 (obtained from Thermo Fisher Scientific, Waltham, MA USA) was used. A Venusil ASB C8 column (purchased from BonnaAgela Technologies, USA), 4.6 x 250 mm, a particle size of 5 µm, a pore size of 150 Å and a surface area of 200 $m^2$/g was used. Further details are summarised in Table C-1.

**Table C-1:** HPLC instrument and detector settings

| HPLC instrument settings | |
|---|---|
| Flow rate | 1 mL/min |
| Injection volume | 20 µL |
| Run time | 50 min |
| **Electrochemical detector settings** | |
| Cell potential settings | ESA 5011A Analytical cell potential settings |
| | Coulometric Electrochemical Detection |
| | Volts: E1: 150mV, E2: +750mV |
| | Guard Cell (EGC ): +350 mV |
| | Detection range: 500 nA Filter: 0.5 seconds |
| | Offset: 0% |
| | Signal output: 0.1 V |

### 2.1.2 Chemicals and reagents

Reagents for the mobile phase and homogenisation solution were purchased from Merck (Pty) Ltd., Johannesburg, South Africa. Monoamine salts and the internal standard, 5-hydroxy-Nω-methyltryptamine oxalate (5-HMT), were purchased from Sigma-Aldrich Pty. Ltd., Johannesburg, South Africa.

#### 2.1.2.1 Preparation of Solution A, monoamine standards, internal standard, and mobile phase

Solution A

Solution A was used as the preparation solvent for all samples. The solution consisted of 0.1 M perchloric acid; 0.5 mM sodium metabisulphite and 0.3 mM Ethylenediaminetetraacetic acid disodium salt. This solution keeps the samples stable by protecting the monoamines from auto-oxidation and facilitating the precipitation of proteins in the biological samples (Viljoen *et al.*, 2018).

# Addendum C

Monoamine standards

Standard stock solution was prepared at a concentration of 100 µg/ml for the analytes detailed in Table 1. Solution A was used as the diluent. From this stock solution, a range of concentrations (5 ng/ml to 200 ng/ml) were prepared to setup a standard calibration curve. Linear standard curves (regression coefficient > 0.99) were prepared in this range. Monoamine concentrations were expressed as ng/g wet weight of tissue. Measurements of monoamine standard reagents used to make up the standard stock solution are summarised in Table C-2.

**Table C-2:** Preparation of the monoamine standard stock solutions made to a final concentration of 100 µg/mL.

| Analyte | Molar mass (g/mol) | Reagent | Molar mass (g/mol) | Mass (g) of reagent dissolved in Solution A |
|---|---|---|---|---|
| NE | 169.18 | l-noradrenaline hydrochloride | 205.64 | 1.22 |
| DOPAC | 168.15 | 3,4-dihydroxyphenylacetic acid | 168.15 | 1.00 |
| DA | 153.18 | 3-hydroxytyramine hydrochloride | 189.64 | 1.24 |
| HVA | 182.18 | homovanillic acid | 182.18 | 1.00 |
| 3-MT | 167.21 | 3-methoxytyramine | 203.67 | 1.22 |
| 5-HT | 176.20 | 5-hydroxytryptamine creatinine sulphate | 405.43 | 2.30 |
| 5-HIAA | 191.19 | 5- Hydroxyindole-3-acetic acid | 191.19 | 1.00 |

Internal standard solution

A stock solution of the internal standard, 5-HMT, with a concentration of 100 µg/ml using solution A as the solvent was made. A final concentration of 1500 ng/mL working internal solution was prepared using Solution A as a diluent.

Mobile phase

Mobile phase was made up of 0.1 M sodium formate buffer, 5 mM sodium 1-heptanesulfonate, 0.17 mM ethylenediaminetetra- acetic acid disodium salt and v/v 5% acetonitrile. The pH of the mobile phase was adjusted to pH 4.00 with ortho-phosphoric acid. The mobile phase was filtered through a 0.22 µm nylon filter before use (Agela Technologies).

### 2.1.3 Sample preparation and analysis of monoamine content

1. The hippocampus and frontal cortex of each rat were dissected out on an ice-cooled glass slab as follows: the base of the brain and olfactory bulb were first removed and the brain bisected into the right and left cerebral hemispheres. The frontal cortex was dissected with the corpus callosum anterior tip being the external limit. The brain was then placed on its ventral side to excise the hippocampus. The brain regions were snap frozen separately in liquid nitrogen and stored at -80°C until analysis.
2. On the day of analysis, the tissue samples were weighed individually and 1 mL of homogenisation solution (solution A) was added to the same tube. The tissue was then ruptured by sonication (2 x 12 seconds at an amplitude of 14 µ) (Keller *et al.*, 1976).
3. The samples were placed in ice for a period of 20 min to complete perchlorate precipitation of protein and extraction of monoamines.
4. Following this, samples were centrifuged at 4°C at 14000 rpm for 20 min. The supernatant was transferred into a 2 mL amber polypropylene tube. A drop (approximately 5 µL) of 10 M potassium acetate was added to the supernatant to adjust its pH to 5.
5. 200 µL of the sample was aliquotted into a clean, transparent 1.5 mL polypropylene tube and 20 µL of the internal standard, 5-HMT, was added to the sample. The final sample was vortexed and then centrifuged for 5 min at 14 000 rpm and transferred to HPLC vial inserts.
6. The peak area data of each sample is collected, divided by the peak area of the internal standard, and converted to a concentration value in ng/ml by factoring in the standard linear calibration curve of each analyte (regression >0.99).

#### 2.1.3.1 Chromotographic information

Table C-3 reports retention times for the respective analytes while Figure 1 shows a typical chromatogram of all the monoamine, their metabolites, and the internal standard.

**Table C-3:** Retention times of the monoamine analytes, their metabolites, and the internal standard.

| Analyte | Retention time (min) |
|---|---|
| NE | ±6.20 |
| DOPAC | ±10.48 |
| DA | ±12.39 |
| 5-HIAA | ±20.43 |
| HVA | ±24.44 |
| 3-MT | ±28.54 |
| 5-HT | ±32.86 |
| Internal standard – 5-HMT | ±42.71 |

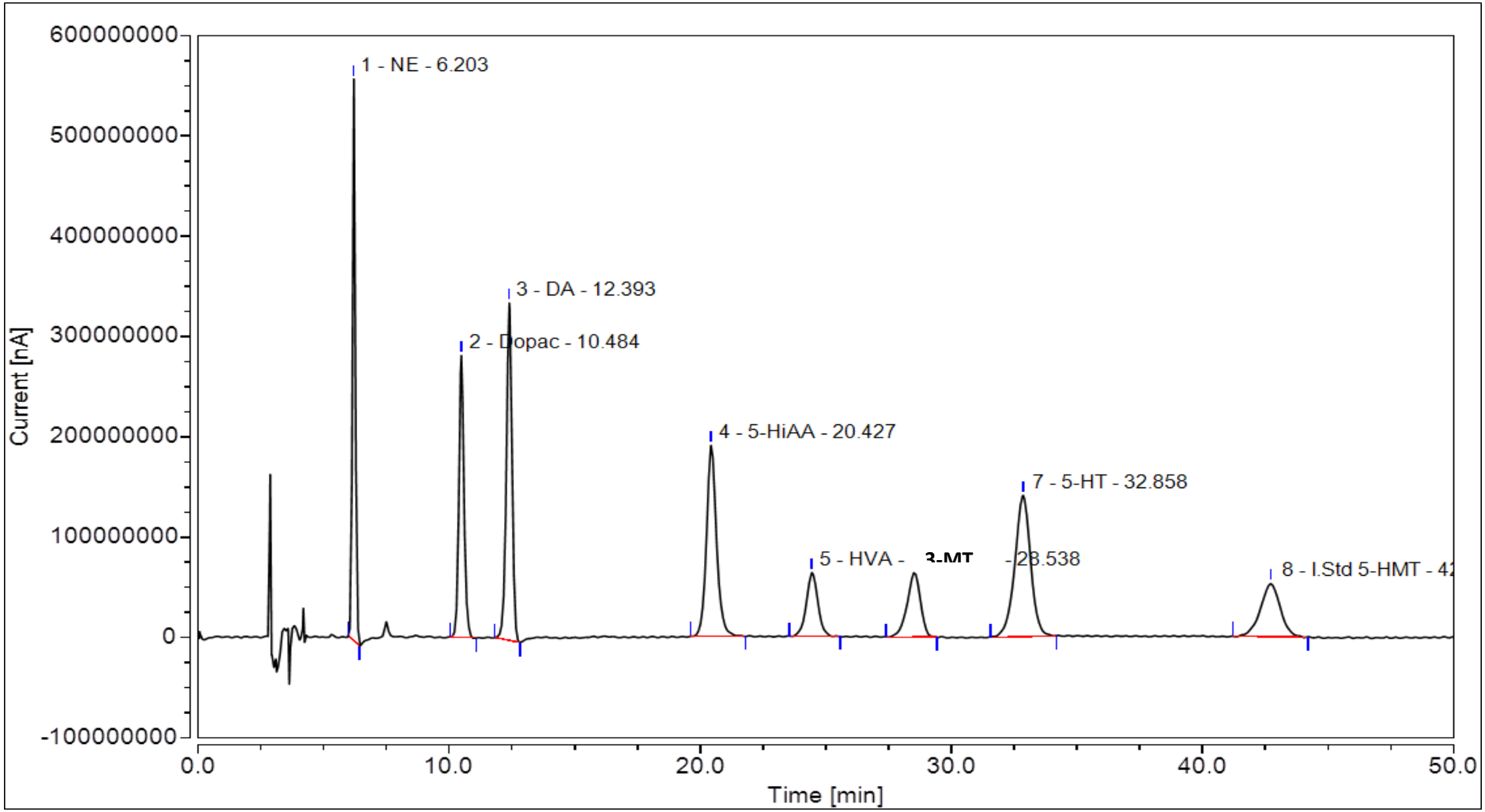


**Figure C-2:** Representative chromatogram of monoamine standards and 200 ng/mL internal standard.

### 2.1.4 Method validation

Method validation is performed prior to starting sample analysis. This is used to verify the suitability of the analytical test system as well as reliability and consistency of the analytical results (Rao, 2018).

#### 2.1.4.1 Validation parameters

The analytical parameters used in this mini method validation are as follows: a) calibration curve (linearity), b) repeatability, c) lower limit of quantification (LLOQ) and d) lower limit of detection (LLOD).

#### 2.1.4.2 Calibration curve (Linearity)

The calibration curves of the analytes were built by plotting peak area ratio of compounds to internal standard at these concentrations: 10, 25, 50, 100, 150, and 200 ng/mL. The sample results were calculated from this. A linear equation must be obtained with a regression coefficient ($R^2$) greater than or equal to 0.99 (Reddy *et al.*, 2018) or greater than 0.95 for endogenous biomolecules (Shabir, 2005). The linear regressions of the analytes and IS are reported in Table 4.

**Table C-4:** Linear regression line equation and coefficient of determination.

| Monoamines | y = mx + c | Coefficient of determination ($R^2$) |
|---|---|---|
| NE | y = 374 441.40x | 0.9988 |
| DA | y = 440 697.43x | 0.9979 |
| DOPAC | y = 320 707.41x + 694 957.30 | 0.9992 |
| HVA | y = 168 634.78x + 119 620.43 | 0.9996 |
| 5-HT | y = 513 707.56x + 160 266.26 | 0.9997 |
| 5-HIAA | y = 440 729.01x + 98 375.67 | 0.9992 |

#### 2.1.4.3 Repeatability

Repeatability measures the precision of the method. This is reported by the %coefficient of

variation (CV) and is determined at 3 concentrations (lowest, middle, and highest concentrations) and using 3 repeats for each value (Huber, 2010). The % CV is determined by dividing the standard deviation of the 3 repetitions by their mean and multiplying this number by 100. The concentrations used to measure repeatability were: 10, 100, 200 ng/mL.

#### 2.1.4.4 Sensitivity

Sensitivity refers to the quantification and detection limits. The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were determined to be 5.0 ng/ml and 10 ng/ml for all the analytes, respectively.

A few unknown peaks are sometimes detected in the samples. This is because biological samples are being analysed and thus other biomolecules can be detected.

### 2.2 ELISA

This section describes the procedures used to measure plasma levels of DBH, CORT, IL-6, TNF-α, and BDNF.

Kits used

1. DBH (Catalogue no: abx256508, Abbexa, Cambridge, UK)
2. CORT (Catalogue no: E-EL-R0269, Elabscience Biotechnology Inc., Wuhan China)
3. IL-6 (Catalogue no: E-EL-R0015, Elabscience Biotechnology Inc., Wuhan China)
4. TNF-α (Catalogue no: E-EL-R0019, Elabscience Biotechnology Inc., Wuhan China)
5. BDNF (Catalogue no: E-EL-R1235, Elabscience Biotechnology Inc., Wuhan China)

#### 2.2.1 DBH

**The reference standard and sample** diluent provided in the kit were used to prepare serial dilutions, the resulting dilution gradient of which was used to plot a calibration (standard) curve. The standard was centrifuged at 10 000 x g for 1 min. To this, 1 mL of reference standard and sample diluent were added and the mixture was left to stand for 10 min. Following this, the mixture was gently inverted several times to facilitate dissolution. This was further mixed with a pipette. This reconstitution produced a working solution of 2000 pg/mL. Figures C-3a – d show the DBH

standard curves.

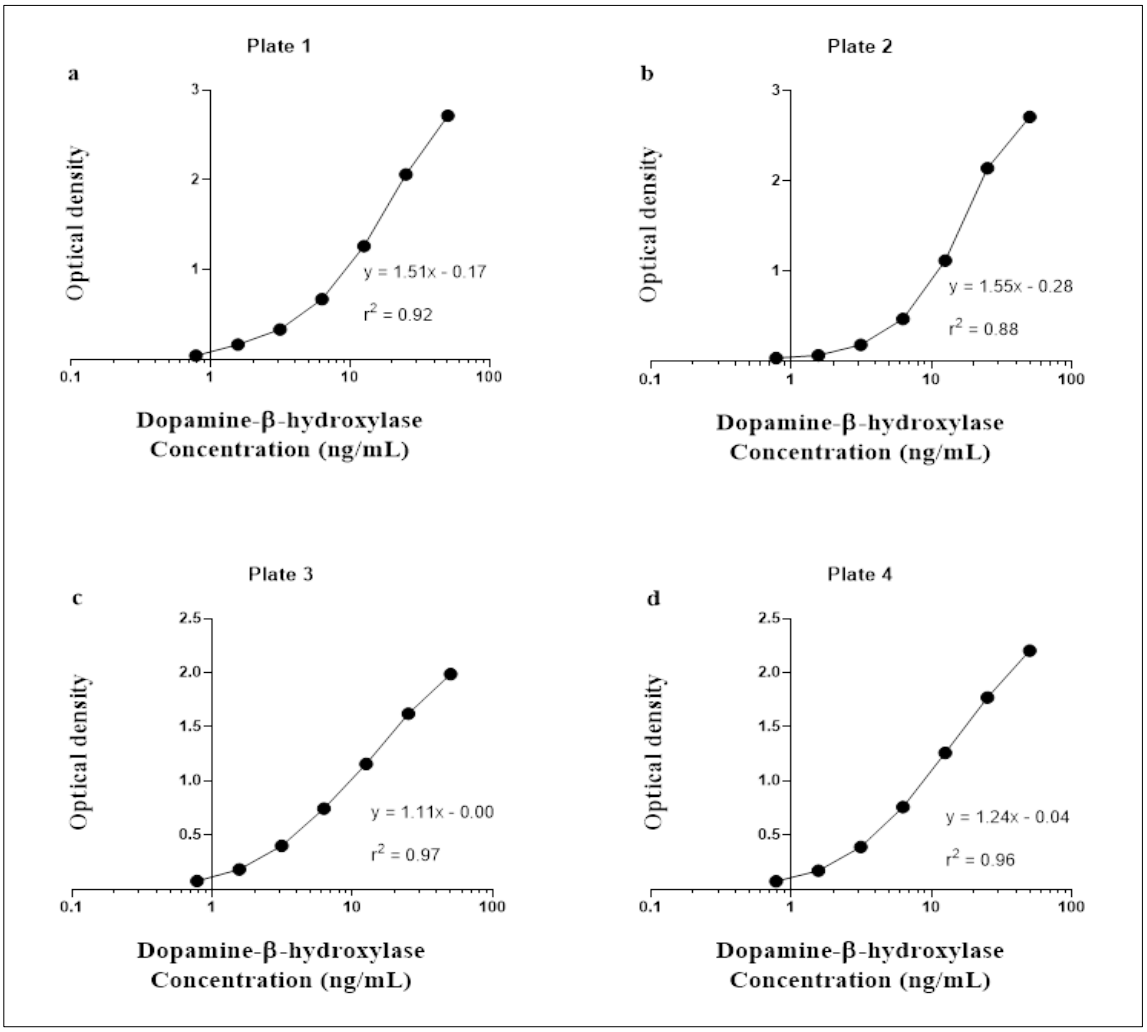


**Figure C-3a – d:** Standard curves of Dopamine-β-hydroxylase (DBH) for each ELISA plate.

### 2.2.2 CORT

The reference standard and sample diluent provided in the kit were used to prepare serial dilutions, the resulting dilution gradient of which was used to plot a standard curve. The standard was centrifuged at 10 000 x g for 1 min. To this, 1 mL of reference standard and sample diluent were added and the mixture was left to stand for 10 min. Following this, the mixture was gently inverted several times to facilitate dissolution. This was further mixed with a pipette. This reconstitution produced a working solution of 200 ng/mL. The standard curves for CORT are shown in Figures C-4a – d.

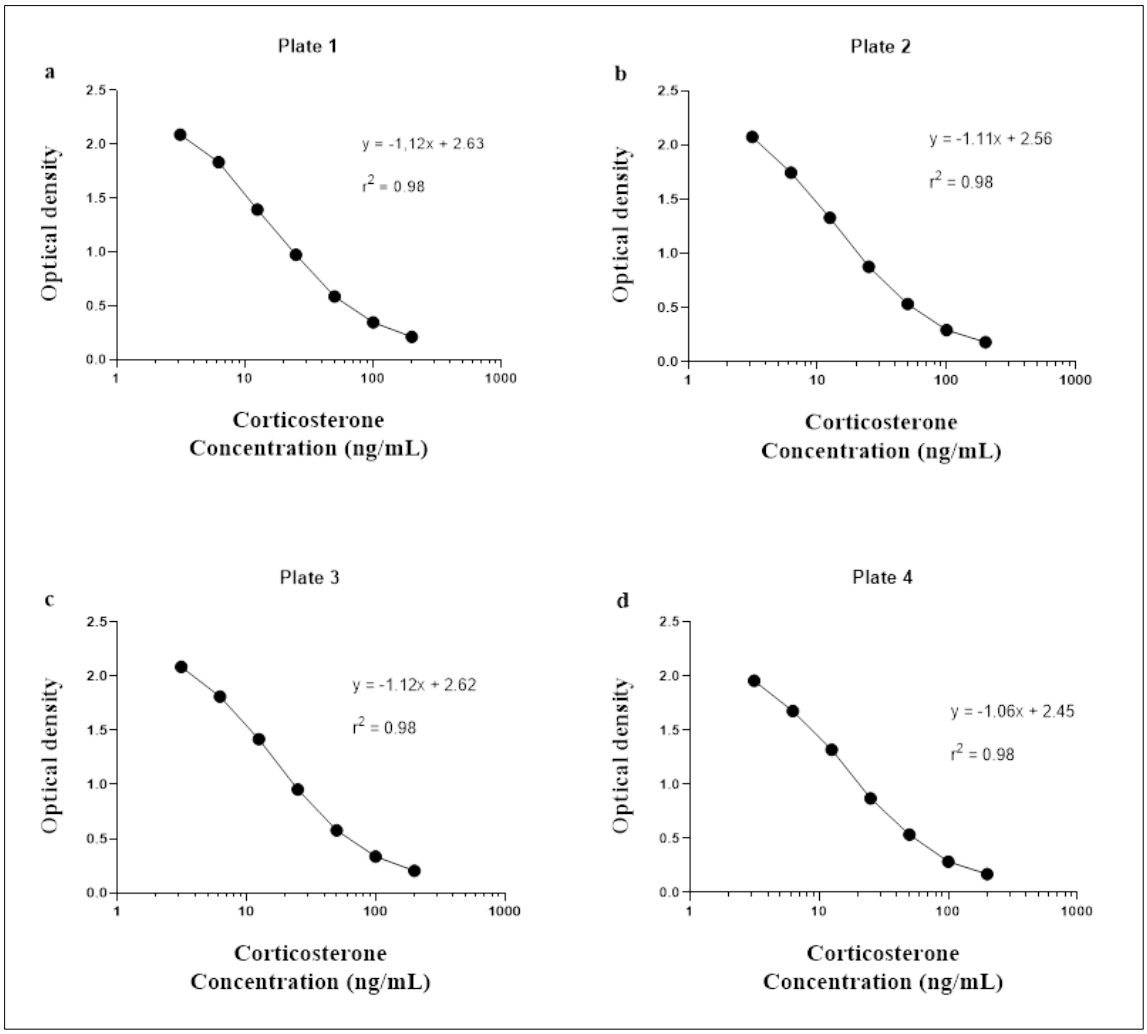


**Figure C-4a – d:** Standard curves of Corticosterone (CORT) for each ELISA plate.

### 2.2.3 IL-6

The reference standard and sample diluent provided in the kit were used to prepare serial dilutions, the resulting dilution gradient of which was used to plot a standard curve. The standard was centrifuged at 10 000 x g for 1 min. To this, 1 mL of reference standard and sample diluent were added and the mixture was left to stand for 10 min. Following this, the mixture was gently inverted several times to facilitate dissolution. This was further mixed with a pipette. This reconstitution produced a working solution of 800pg/mL. Figure C-5a – d shows the standard curves for IL-6.

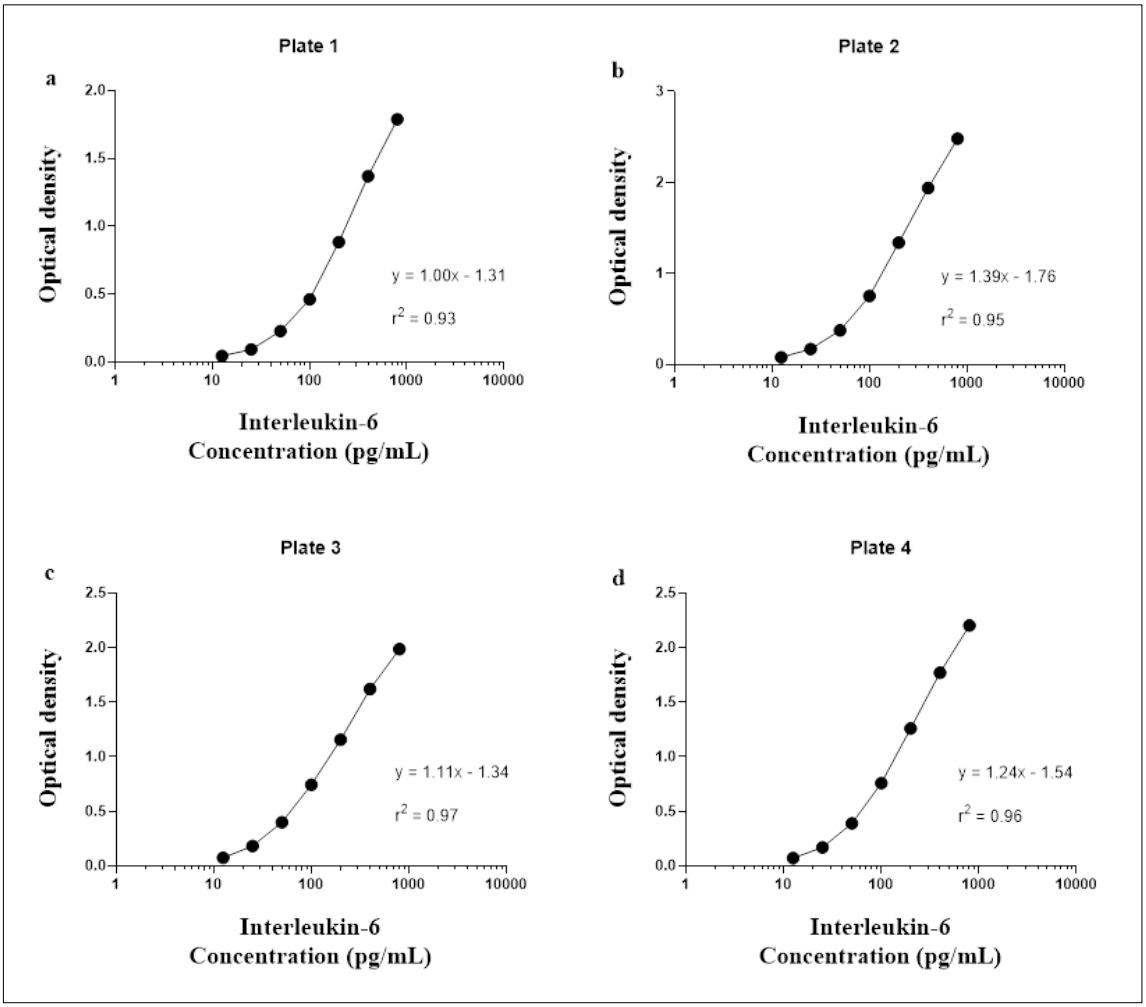


**Figure C-5a – d:** Standard curves of Interleukin-6 (IL-6) for each ELISA plate.

### 2.2.4 TNF-α

The reference standard and sample diluent provided in the kit were used to prepare serial dilutions, the resulting dilution gradient of which was used to plot a standard curve. The standard was centrifuged at 10 000 x g for 1 min. To this, 1 mL of reference standard and sample diluent were added and the mixture was left to stand for 10 min. Following this, the mixture was gently inverted several times to facilitate dissolution. This was further mixed with a pipette. This reconstitution produced a working solution of 5000pg/mL. The standard curves of TNF-α are shown in Figures C-6a – d.

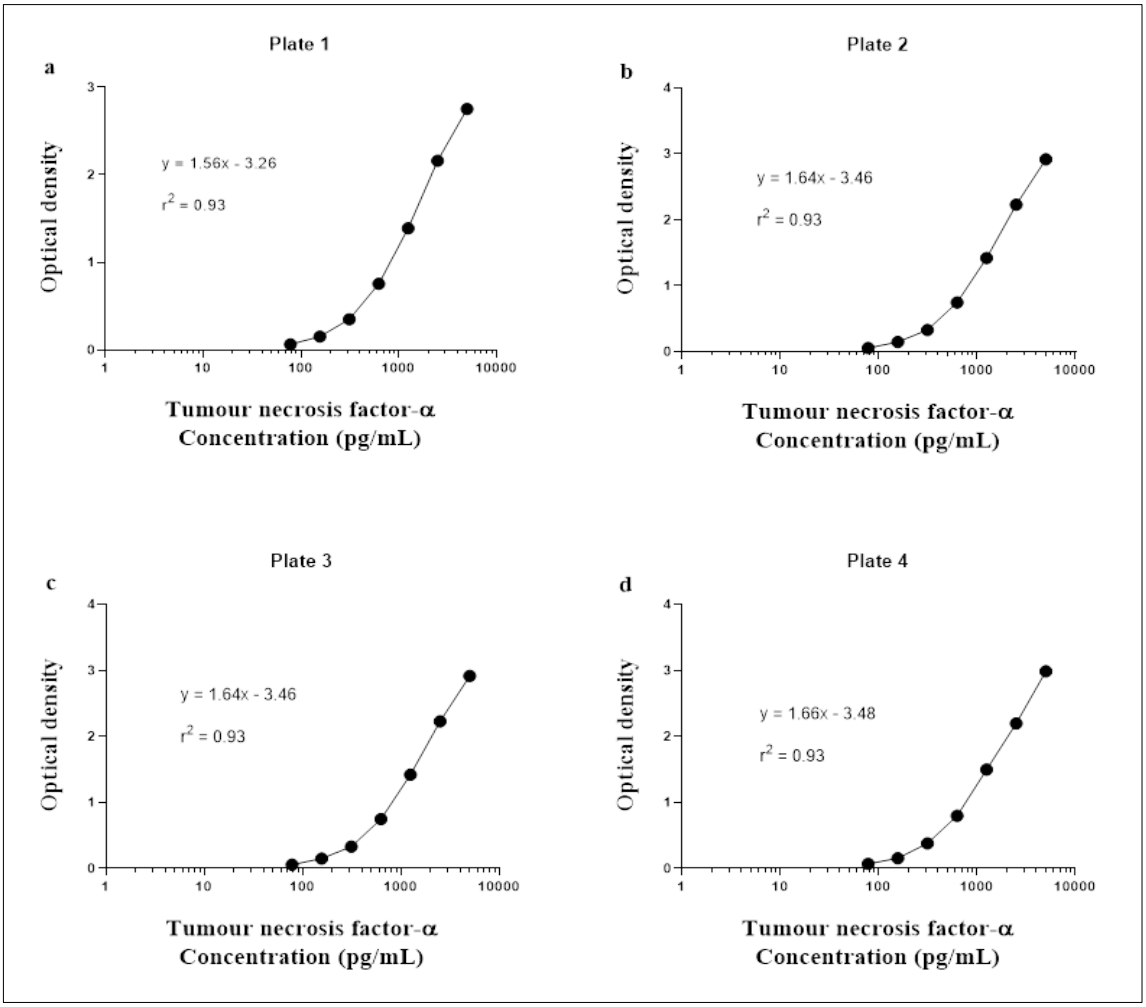


**Figure C-6a – d:** Standard curves of Tumour necrosis factor alpha (TNF-α) for each ELISA plate.

### 2.2.5 BDNF

The reference standard and sample diluent provided in the kit were used to prepare serial dilutions, the resulting dilution gradient of which was used to plot a standard curve. The standard was centrifuged at 10 000 x g for 1 min. To this, 1 mL of reference standard and sample diluent were added and the mixture was left to stand for 10 min. Following this, the mixture was gently inverted several times to facilitate dissolution. This was further mixed with a pipette. This reconstitution produced a working solution of 2000pg/mL. Figures C-7a – d show the standard curves for BDNF.

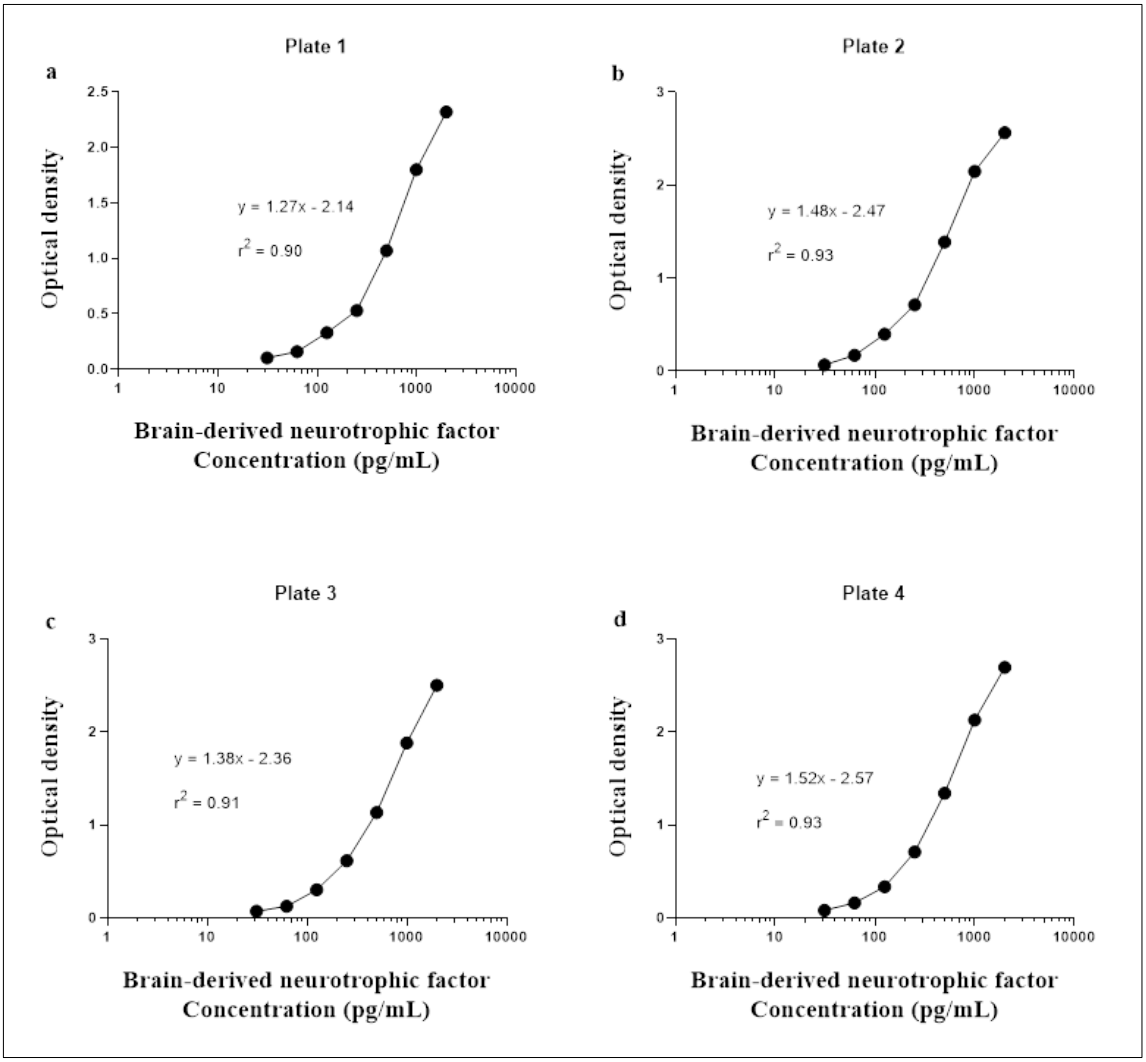


**Figure C-7a – d:** Standard curves of Brain-derived neurotrophic factor (BDNF) for each ELISA plate.

**Table C-5:** Kit sensitivity, detection range, and reproducibility

| Biomarker | Sensitivity | Detection range | Reproducibility |
|---|---|---|---|
| DBH | < 0.39 ng/mL | 0.78 ng/mL – 50 ng/mL | (not listed) |
| CORT | 1.88 ng/mL | 3.13 – 200 ng/mL | coefficient of variation is < 10% |
| IL-6 | 7.50 pg/mL | 12.50 – 800 pg/mL | coefficient of variation is < 10% |
| TNF-α | 46.88 pg/mL | 78.13 – 5000 pg/mL | coefficient of variation is < 10% |
| BDNF | 18.75 pg/mL | 31.25 – 2000 pg/mL | coefficient of variation is < 10% |

These kits were developed and validated by the manufacturer for the *in vitro* quantification of these markers in rat serum and plasma blood tissue. The following sections describe the materials and procedure used to construct calibration curves for each kit.

### 2.2.6 Assay procedure

The assay procedures for all the kits are similar and so a general description of the ELISA procedure is described below. All steps are performed at room temperature (20-25$^0$C) as per the manufacturer's instructions.

1. Trunk blood was collected in pre-chilled, 4 mL vacutainer tubes (Vacuette®) containing $K_3$EDTA solution as anticoagulant. The blood was centrifuged at 1000 × g at 4°C for 15 min. The supernatant (plasma) was collected, fixed in liquid nitrogen and stored at −80°C until the day of analysis. The samples were used within 3 months of being frozen.
2. On the day of analysis, the kits and samples were brought out of cold storage and brought to room temperature (20-25°C) before use.
3. The reagents supplied in the kit were prepared as per the manufacturer's protocol.
4. Standards or samples were added to the micro ELISA plate wells and combined with the relevant antibody. The plate was sealed and incubated at 37°C for the prescribed time.
5. After incubation, the liquid from each well was removed.
6. Biotinylated detection antibody was added to each well followed by an incubation period at 37°C.
7. After incubation, the plate was aspirated and washed to remove free components.
8. Avidin-horseradish peroxidase (HRP) conjugate was added to each well. The plate was covered then incubated at 37°C.
9. This was followed by another "aspirate and wash" sequence.
10. The substrate solution is added to each well and the plate was covered and incubated at 37°C away from light.
11. Stop solution was added to each well. This solution terminates the enzyme-substrate reaction. The colour change that occurred in the incubation stage of step 10 was measured spectrophotometrically using a micro-plate reader set to 450 nm wavelength.

12. The concentration of the analyte in the samples was determined by comparing the optical density value of each sample-containing well to the standard curve.

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# APPENDIX A: CO-AUTHOR APPROVAL LETTERS

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17 November 2020

**The Director: Higher Degrees Administration**
**North-West University**
**11 Hoffman Street**
**Potchefstroom**
**2520**
**SOUTH AFRICA**

**Dear Sir/Madam,**

**RE: CO-AUTHOR PERMISSION TO SUBMIT CHAPTER 3 OF THIS THESIS FOR EXAMINATION PURPOSES**

**Hereby I, Prof Brian H. Harvey, of the above institution, a co-author of the manuscript titled "Flinders Sensitive Line rats exposed to post weaning isolation rearing are an animal model of treatment resistant depression", which is included in Chapter 3 of this thesis, give permission for this work to be submitted for examination purposes.**

**Regards,**

# Appendix A: Co-Author Approval Letters

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**Regards,**

# Appendix A: Co-Author Approval Letters

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Dear Sir/Madam,

**RE: CO-AUTHoR PERMISSION TO SUBMIT CHAPTER 3 OF THIS THESIS FOR EXAMINATION PURPOSES**

Hereby I, Dr Marisa Moller-Wolmarans, of the above institution, a co-author of the manuscript titled "Flinders Sensitive Line rats exposed to post weaning isolation rearing are an animal model of treatment resistant depression", which is included in Chpater 3 of this thesis, give permission for this work to be submitted for examination purposes.

Regards,

1

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Dear Sir/Madam,

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Regards,

1

# APPENDIX B: CONFIRMATION OF SUBMISSION OF MANUSCRIPT *A* TO JAD

**From:** "Journal of Affective Disorders" <em@editorialmanager.com>
**To:** "Brian Harvey" <brian.harvey@nwu.ac.za>
**Date:** 2020/10/29 05:00
**Subject:** Submission to Journal of Affective Disorders - manuscript number

*This is an automated message.*

Manuscript Number: JAFD-D-20-01865
Flinders Sensitive Line rats exposed to post weaning isolation rearing are an animal model of treatment resistant depression

Dear Harvey,

Your above referenced submission has been assigned a manuscript number: JAFD-D-20-01865.

To track the status of your manuscript, please log in as an author at https://www.editorialmanager.com/jafd/, and navigate to the "Submissions Being Processed" folder.

Thank you for submitting your work to this journal.

Kind regards,
Journal of Affective Disorders

More information and support

You will find information relevant for you as an author on Elsevier's Author Hub: https://www.elsevier.com/authors

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For further assistance, please visit our customer service site: https://service.elsevier.com/app/home/supporthub/publishing/
Here you can search for solutions on a range of topics, find answers to frequently asked questions, and learn more about Editorial Manager via interactive tutorials. You can also talk 24/7 to our customer support team by phone and 24/7 by live chat and email

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: https://www.editorialmanager.com/jafd/login.asp?a=r). Please contact the publication office if you have any questions.

# APPENDIX C: LETTER OF ETHICAL APPROVAL

Private Bag X6001, Potchefstroom
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**Health Sciences Ethics Office for Research, Training and Support**

**Animal Care, Health and Safety in Research Ethics Committee (AnimCare)**
Tel: 018 299 2234
Email: Tiaan.Brink@nwu.ac.za

Prof BH Harvey
PharmaCen

08 June 2018

Dear Prof Harvey

## APPROVAL OF YOUR APPLICATION BY THE ANIMCARE COMMITTEE OF THE FACULTY OF HEALTH SCIENCES

**Ethics number: NWU-00150-18-S5**

Kindly use the ethics reference number provided above in all future correspondence or documents submitted to the administrative assistant of the Animal Care, Health and Safety in Research Ethics Committee (AnimCare).

**Study title: Development and validation of an animal model of treatment-resistant depression with psychotic features**

**Study leader: Prof Brian Harvey**

**Student: Ms K Mncube**

**Application type: Single study**

| **Project Category** *(impact on animal wellbeing)* | **NA** | **0** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|---|---|
| | | | | | | | X |

**Expiry date: 30 June 2019 (Monitoring reporting six monthly i.e. end of February and end of June)**

You are kindly informed that after review by the AnimCare committee, Faculty of Health Sciences, North-West University, your ethics approval application has been successful and was determined to fulfil all requirements for approval. Your study is approved for a year and may commence from 08/06/2018. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation. A monitoring report should be submitted two months prior to the reporting dates as indicated i.e. annually for Category 0-4 studies, six-monthly for category 5 studies, to ensure timely renewal of the study. A final report must be provided at completion of the study or the AnimCare committee, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimMonitoring@nwu.ac.za. Annually, a number of studies may be randomly selected for an internal audit.

The AnimCare committee, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the AnimCare committee, Faculty of Health Sciences prior to implementing these changes. These requests should be submitted to Ethics-AnimCare@nwu.ac.za with a cover letter with a specific subject title indicating "Amendment request: NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted. The amendments made should be indicated in yellow highlight in the amended documents (or in the fillable MSWord format application forms where a yellow highlighter may not be visible, change the text colour to red). The *e-mail*, to which you attach the documents that you send, should have a *specific subject line* indicating that it is an amendment request e.g. "Amendment request: NWU-XXXXX-XX-XX". This e-mail should indicate the nature of the amendment. This submission will be handled via the expedited process.

Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form to Ethics-AnimCareIncident-SAE@nwu.ac.za. The *e-mail*, to which you attach the documents that you send, should have a specific subject line indicating that it is a notification of a serious adverse event or incident in a specific project e.g. "SAE/Incident notification: NWU-XXXXX-XX-XX".

Please note that the AnimCare committee, Faculty of Health Sciences has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research. The AnimCare committee, Faculty of Health Sciences reserves the right to visit sites where approved studies will be conducted and any animal housing facility under the authority of NWU as often as it deems necessary, either announced or unannounced.

The AnimCare committee, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the South African National Standard (SANS) document 10386:2008 entitled, "The care and use of animals for scientific purposes", the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimCare@nwu.ac.za.

Yours sincerely

Prof Christiaan B Brink

Chair: AnimCare

Prof Minrie Greeff

Head: Ethics Office

Current details: (1300923002) G:\My Drive\9. .5\.Ethics\NWU-00150-18-S59.1.5.4.1_Approval_letter_AnimCare.docm
08 June 2018

File reference: 9.1.5.4.1

# APPENDIX D: ANIMAL MONITORING SHEETS

**AnimCare 06-01a v4.10** — **Monitoring Sheet for Animal Welfare During Study**

| | | | | |
|---|---|---|---|---|
| **Study title:** | Development and validation of an animal model of treatment-resistant depression with psychotic features | | | **Year:** 2018 |
| **Ethics no.:** | NWU-00150-18-S5 | **Project head:** Prof. B.H. Harvey | **Observer / student:** Ms K. Mncube | **Animal ID:** |

| Parameter | | Score | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Appearance | Normal | 0 | | | | | | | | | | | | | | | | | | | | | |
| | Lack of grooming | 1 | | | | | | | | | | | | | | | | | | | | | |
| | Dull coat, ocular/nasal discharge | 2 | | | | | | | | | | | | | | | | | | | | | |
| | Piloerection, hunched up | 3 | | | | | | | | | | | | | | | | | | | | | |
| Food & water intake | Normal | 0 | | | | | | | | | | | | | | | | | | | | | |
| | <5% weight loss | 1 | | | | | | | | | | | | | | | | | | | | | |
| | Noted intake, 5-15% weight loss | 2 | | | | | | | | | | | | | | | | | | | | | |
| | No food or water intake | 3 | | | | | | | | | | | | | | | | | | | | | |
| Clinical signs | Normal | 0 | | | | | | | | | | | | | | | | | | | | | |
| | Slight changes | 1 | | | | | | | | | | | | | | | | | | | | | |
| | Respiratory increase ↑ 30% | 2 | | | | | | | | | | | | | | | | | | | | | |
| | Respiratory increase ↑ 50% | 3 | | | | | | | | | | | | | | | | | | | | | |
| Natural behaviour | Normal | 0 | | | | | | | | | | | | | | | | | | | | | |
| | Minor change | 1 | | | | | | | | | | | | | | | | | | | | | |
| | Less mobile alert, isolated | 2 | | | | | | | | | | | | | | | | | | | | | |
| | Vocalisation, restless or still | 3 | | | | | | | | | | | | | | | | | | | | | |
| Provoked behaviour | Normal | 0 | | | | | | | | | | | | | | | | | | | | | |
| | Minor depression | 1 | | | | | | | | | | | | | | | | | | | | | |
| | Moderate change | 2 | | | | | | | | | | | | | | | | | | | | | |
| | Reacts violently/weakly, precomatose | 3 | | | | | | | | | | | | | | | | | | | | | |
| | TOTAL SCORE | 0-15 | | | | | | | | | | | | | | | | | | | | | |
| Project-specific | Criterion 1 | | | | | | | | | | | | | | | | | | | | | | |
| | Criterion 2 | | | | | | | | | | | | | | | | | | | | | | |
| | Criterion 3 | | | | | | | | | | | | | | | | | | | | | | |
| | Criterion 4 | | | | | | | | | | | | | | | | | | | | | | |
| Other | Observation and/or comment (tick box ☐ if written on reverse side) | | 1☐ reverse | 2☐ reverse | 3☐ reverse | 4☐ reverse | 5☐ reverse | 6☐ reverse | 7☐ reverse | 8☐ reverse | 9☐ reverse | 10☐ reverse | 11☐ reverse | 12☐ reverse | 13☐ reverse | 14☐ reverse | 15☐ reverse | 16☐ reverse | 17☐ reverse | 18☐ reverse | 19☐ reverse | 20☐ reverse | 21☐ reverse |
| Decision | ✓ = normal / ? = monitor carefully / ! = seek advice / ✕ = intervene immediately | | | | | | | | | | | | | | | | | | | | | | |
| Signature (please sign/initialise with each observation per column) | | | | | | | | | | | | | | | | | | | | | | | |
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |

00 - 04 = Normal
05 - 09 = monitor carefully, consider intervention
10 - 13 = Suffering, provide relief, observe regularly. Seek opinion from technologist as per callout sheet. Consider humane euthanasia.
14 - 15 = Sever pain; intervene immediately per humane endpoint, reconsider experimental protocol.

**Observations and/or comments, corresponding to the column on the front page of the monitoring sheet *(see reverse side)***

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# Addendum D: Animal monitoring sheets

| | |
|---|---|
| Rat FST: | **Rat Forced Swim Test Animal Monitoring Record** |
| Study title: | **Development and validation of an animal model of treatment-resistant depression with psychotic features** Year: 2018 |
| Ethics no.: | No. Project head: Prof BH Harvey Observer / student: Miss K Mncube |

**FST Animal Monitoring *(recorded time of 7 minutes)***

| Animal ID: | Condition of animal prior to FST: | Observed abnormal behaviour during FST (Y/N)? Description of abnormal behaviour (e.g. complete lack of swimming behaviour considered abnormal): | Was animal removed prior to completion time (Y/N)? Reason for removal (e.g. drowning): |
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Study title: **Development and validation of an animal model of treatment-resistant depression with psychotic features**

**2 hours post-prepulse inhibition test monitoring sheet for excessive stress and / or behavioural abnormalities:**

| Parameter | Animal ID | Score | |
|---|---|---|---|
| **Appearance** | Normal | 0 | |
| | Lack of grooming | 1 | |
| | Dull coat, nasal/ocular discharge | 2 | |
| | Piloerecton, hunched up | 3 | |
| **Clinical signs** | Normal | 0 | |
| | Slight changes in respiratory and cardiac rate (indicative of some distress) | 1 | |
| | Respiration 30%, Tremors (indicative of distress) | 2 | |
| | Respiration 50%, Convulsions (indicative of severe distress) | 3 | |
| **Natural behaviour** | Normal | 0 | |
| | Minor changes (lack of grooming etc.) | 1 | |
| | Less mobile / altert, isolated | 2 | |
| | Vocalization, restless or still / immobile | 3 | |
| **Provoked behaviour** | Normal | 0 | |
| | Minor depression (isolation / lack of grooming) | 1 | |
| | Moderate change | 2 | |
| | Reacts violently / weakly, precomatose | 3 | |
| **TOTAL** | | | |
| **Signature** | | | |

0-4 = Normal

5-9 = Monitor carefully, consider intervention.

10-13 = Suffering, provide relief, observe regularly. Seek second opinion from technologist as per call out sheet. Consider humane euthanasia.

14-15 = Severe pain. Intervene immediately per humane endpoint, reconsider experimental protocol.

Sgnatures

Print name _____ Sign _____

Study director sign _____ Date _____

# APPENDIX E: CONGRESS PROCEEDINGS

Findings from this study were presented at an international congress (poster presentation).

1. K Mncube, M Möller-Wolmarans*, Brian H Harvey (2019). Exploring a gene-x-environment model of treatment resistant depression: behavioural studies and response to fluoxetine treatment. The 32nd Congress of the European College of Neuropsychopharmacology, Copenhagen, Denmark (7 – 10 September 2019).

   Abstract available at: https://doi.org/10.1016/j.euroneuro.2019.09.764

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