



# **Evaluating the neuropsychiatric properties of efavirenz in an inflammatory model of schizophrenia**

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*Vir my ewige held: Dadda*

*Kobus Pieters*

*18 Julie 1972 – 9 Oktober 2014*

God of creation  
There at the start  
Before the beginning of time  
With no point of reference  
You spoke to the dark  
And fleshed out the wonder of light

And as You speak  
A hundred billion galaxies are born  
In the vapor of Your breath the planets form  
If the stars were made to worship so will I  
I can see Your heart in everything You've made  
Every burning star  
A signal fire of grace  
If creation sings Your praises so will I

God of Your promise  
You don't speak in vain  
No syllable empty or void  
For once You have spoken  
All nature and science  
Follow the sound of Your voice

And as You speak  
A hundred billion creatures catch Your breath  
Evolving in pursuit of what You said  
If it all reveals Your nature so will I  
I can see Your heart in everything You say  
Every painted sky  
A canvas of Your grace  
If creation still obeys You so will I

If the stars were made to worship so will I  
If the mountains bow in reverence so will I  
If the oceans roar Your greatness so will I  
For if everything exists to lift You high so will I  
If the wind goes where You send it so will I  
If the rocks cry out in silence so will I  
If the sum of all our praises still falls shy  
Then we'll sing again a hundred billion times

God of salvation  
You chased down my heart  
Through all of my failure and pride  
On a hill You created  
The light of the world  
Abandoned in darkness to die

And as You speak  
A hundred billion failures disappear  
Where You lost Your life so I could find it here  
If You left the grave behind You so will I  
I can see Your heart in everything You've done  
Every part designed in a work of art called love  
If You gladly chose surrender so will I  
I can see Your heart  
Eight billion different ways  
Every precious one  
A child You died to save  
If You gave Your life to love them so will I

Like You would again a hundred billion times  
But what measure could amount to Your desire  
You're the One who never leaves the one behind

***So will I – Hillsong United***

## TABLE OF CONTENTS

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<b>ACKNOWLEDGMENTS</b>	i
<b>ABSTRACT</b>	iv
<b>LIST OF FIGURES</b>	vi
<b>LIST OF TABLES</b>	xiii
<b>LIST OF ABBREVIATIONS</b>	xv
<b>GLOSSARY</b>	xxiii
<b>CHAPTER 1</b>	1
INTRODUCTION	1
1.1    Dissertation approach and layout	1
1.2    Problem statement	2
1.3    Study questions	5
1.4    Study objectives	6
1.5    Hypothesis	7
1.6    Project layout	7
1.7    Expected outcomes	14
1.8    Ethical considerations	15
References	17
<b>CHAPTER 2</b>	26
LITERATURE REVIEW	26
1.1    Human immunodeficiency virus, Acquired Immune Deficiency Syndrome & Highly active anti-retroviral therapy.	26
1.2    Efavirenz	27

1.2.1	Efavirenz abuse and mechanism of efavirenz-related neuropsychiatric effects	28
1.3	Substance-induced psychotic disorder, schizophrenia and addiction	31
1.3.1	Substance-induced psychotic disorder	31
1.3.1.1	Schizophrenia	31
1.3.2	Drug abuse and addiction	33
1.4	Neuroanatomy and neurochemistry	33
1.4.1	Dopamine	34
1.4.1.1	Dopamine transporter	37
1.4.1.2	Dopamine-and- cyclic adenosine monophosphate -regulated phosphoprotein (with a molecular weight of 32kD)	38
1.4.2	Serotonin	40
1.5	Neurodevelopmental hypothesis	42
1.6	Inflammation	43
1.7	The role of the cerebellum in schizophrenia and addiction	46
1.8	Neuroplasticity	47
1.8.1	c-Fos	48
1.9	Hypothalamic-pituitary-adrenal axis	51
1.10	Oxidative stress	52
1.10.1	N-acetylcysteine	54
1.11	Animal models	57
1.11.1	Maternal immune activation	60
1.11.2	Behavioural analysis	61
	Synopsis	63

References	64
<b>CHAPTER 3</b>	107
MANUSCRIPT	107
1. Introduction	111
2. Materials and methods	114
2.1 Statement on ethics	114
2.2 Animals	114
2.3 Study design	114
2.4 Drugs and drug exposure protocol	117
2.5 Body weight	118
2.6 Behavioural analysis	118
2.7 Peripheral and neurochemical analysis	120
2.8 Statistical analysis	122
3. Results	123
3.1 Body weight	123
3.2 Behavioural analysis	123
3.3 Peripheral analysis	130
3.4 Neurochemical analysis	132
4. Discussion	138
References	147
<b>CHAPTER 4</b>	164
CONCLUSION	164
1.1 Concluding remarks	164

1.2	Study outcomes	173
1.3	Limitations and future recommendations	175
	References	176
<b>ADDENDUM A</b>		180
	ETHICS APPROVAL LETTER	180
<b>ADDENDUM B</b>		182
	PRE-NATAL AND POST-NATAL EXPERIMENTAL PROTOCOLS	182
	PRE-NATAL PROTOCOL	182
	Saline and lipopolysaccharide administration	182
	POST-NATAL PROTOCOL	187
	Conditioned place preference	188
	Locomotor activity	191
	Pre-pulse inhibition	192
	References	213
<b>ADDENDUM C</b>		216
	PERIPHERAL- AND NEUROCHEMICAL ANALYSIS	216
	PERIPHERAL ANALYSIS	220
	Corticosterone	220
	Glutathione	226
	NEUROCHEMICAL ANALYSIS	231
	c-Fos	232
	Dopamine transporters	237

Phosphoprotein phosphatase-1 regulatory subunit 1B	247
References	257
<b>ADDENDUM D</b>	259
AUTHOR GUIDELINES	259
Frontiers in Psychiatry	259
<b>ADDENDUM E</b>	279
LETTERS OF CONSENT TO SUBMIT MANUSCRIPT	280

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## ABSTRACT

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Since the inception of the human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) epidemic, a collaborative effort between all branches of medicine contributed towards the establishment of highly active antiretroviral therapy (HAART). Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor, used in combination treatment as part of HAART, presents with neuropsychiatric effects, possibly contributing towards its abuse potential. The impact of EFV abuse on mental health remains equivocal and holds the possibility of addiction or a substance-induced psychotic disorder (SIPD) development. Drug addiction is a chronic relapsing disorder, primarily characterized by a three-staged cycle, viz. intoxication, withdrawal and craving. Patients with a SIPD experience psychotic symptoms after sudden intoxication or withdrawal of addictive drugs and may develop schizophrenia (SCZ) later in life. SCZ is a debilitating disorder where patients experience a combination of positive-, negative- and cognitive symptoms. Specific brain regions (frontal cortex (FC), striatum and possibly the cerebellum) and neurological pathways implicated in the respective disorders may be partially overlapping which may explain why abusive substances may contribute towards the development of psychotic disorders. EFV exerts a variety of mechanisms which may contribute towards the development of these respective disorders, viz. an affinity for the serotonin  $2A$  receptors and dopamine transporters (DAT) as well as the ability to alter regional brain monoamines and redox-inflammatory pathways. Pre-natal inflammation has been implicated in the pathophysiology of psychotic disorders and may contribute towards drug abuse later in life. Therefore, by simulating maternal infection in rodent dams via a bacterial endotoxin, lipopolysaccharide (LPS), a neurodevelopmental model of SCZ as well as its effects on drug abuse later in life can be established. Moreover, there are, no available treatment platform for EFV abuse (with or without contributory factors i.e. pre-natal inflammation) as well as its sequelae. N-acetylcysteine (NAC), a glutathione (GSH) precursor, may be a viable option as it has the capacity to modulate neurotransmission, inflammation and redox homeostasis. The association between psychotic disorders, EFV abuse and its treatment demands further investigation. This study aimed to investigate addictive- (by utilising the conditioned place preference (CPP) paradigm) and psychotic-like (by measuring locomotor activity and pre-pulse inhibition (PPI)) behaviours in rats after pre-natal exposure to bacterial LPS and/or post-natal EFV exposure, and its response to treatment with NAC.

This study was approved (Ethics approval number: NWU-00162-18-S5) by the AnimCare animal research committee (NHREC reg. no. AREC-130913-015) North West University (NWU). All experimental animals used in this study 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 at the NWU. Pregnant Sprague-Dawley rats (12/group) were exposed to either subcutaneous (SC) saline or 100  $\mu$ g/kg LPS on gestational day 15/16. Male pups (n=96)

born from these dams were then randomized into 8 groups (12/group). Starting on post-natal day (PND) 48 all rats were subjected to the CPP paradigm in a drug-free state to establish preference. From PND 49 until PND 54, exposure conditioning (in the CPP paradigm) was performed on alternative days for six days with either olive oil (vehicle) or 5 mg/kg EFV. On PND 55 the first CPP, locomotor activity and PPI were determined. Thereafter, rats received either saline (vehicle) or 100 mg/kg NAC SC for 14 days (PND 56-69). EFV conditioning and behavioural analyses were repeated as per the pre-treatment methodology on PND 70 and 71. The second CPP, locomotor activity and PPI were determined again on PND 72. On PND 73 rats were euthanised and plasma corticosterone (CORT) and GSH, as well as cerebellar c-Fos and striatal and frontal-cortical DAT & phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B)), were analysed.

Sub-acute EFV (5 mg/kg) had no addictive-like (CPP) or SCZ-like, (locomotor activity and %PPI) behaviour (PND 55 and 72) compared to control groups. All peripheral (CORT and GSH) and neurochemical (c-Fos, DAT and PPP1R1P) biomarkers were in accordance and remained unchanged in the group exposed to sub-acute EFV alone. Pre-natal LPS (100 µg/kg) exposure significantly decreased the time spent in the drug-paired compartment (CPP), increased locomotor activity and induced significant %PPI deficits compared to control groups on PND 55. Pre-natal exposure to LPS decreased striatal DAT which were in accordance with the positive SCZ-like symptoms (hyper-locomotion and PPI deficits) observed in the respective groups. These alterations became more apparent in the LPS+EFV groups. The LPS+EFV groups presented with hyper-locomotion and aversive behaviour in the CPP paradigm as well as %PPI deficits on PND 55. LPS+EFV also induced a significant decrease in striatal PPP1R1B and DAT and an increase in plasma CORT and cerebellar c-Fos compared to control groups. No differences were observed in frontal cortical PPP1R1B and DAT across all treatment groups. On PND 72, no %PPI deficits were observed in any groups. Furthermore, on PND 72, the locomotor activity and the striatal PPP1R1B level of the LPS+EFV group correlated with the level of the control group. Literature indicate that, NAC's therapeutic efficacy is based on its capacity to regulate GSH synthesis, however this was not observed in the LPS+EFV group. Therefore, NAC proved to be futile in this study and other mechanisms were considered such as the effects of CORT on dopamine transmission.

To conclude, maternal LPS induced psychotic-like behaviour in off-spring, but not addictive-like behaviour, while post-natal EFV did not induce addictive- or psychotic-like behaviour. The behavioural (hyper-locomotion and %PPI deficits), peripheral (hyper-CORT) and neurochemical (decreased striatal PPP1R1B) alterations induced by LPS became more apparent after EFV exposure. NAC was not a viable treatment with regard to any of the bio-behavioural changes following EFV, LPS or LPS+EFV exposure. This study ultimately highlights the risks of EFV abuse in predisposed individuals.

## LIST OF FIGURES

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### CHAPTER 1

- Figure 1 A-B: A visual diagram of the study design as discussed above. A is the pre-natal saline exposure section and B the pre-natal lipopolysaccharide (LPS) exposure section. 10

### CHAPTER 2

- Figure 1: Proposed mechanism of efavirenz (EFV) to induce central nervous system adverse effects (Apostolova et al., 2015). 1. EFV causes an increase in interleukin (IL)-1 $\beta$  and tumour necrosis factor (TNF)- $\alpha$  (pro-inflammatory cytokines) (O'Mahony et al., 2005). 2. A correlation between an increase in serotonin (5-HT) levels and a decrease in the activity of tryptophan 2,3-dioxygenase (TDO) (Cavalcante et al., 2010). 3. EFV has an affinity for the 5-HT receptors and shows partial agonist activity (Gatch et al., 2013). 4. EFV promotes oxidative stress (Adjene et al., 2010, Brown et al., 2014). 5. Creatine kinase (CK) inhibition in cerebellum, cortex, striatum, and hippocampus (Streck et al., 2008) which could result in cognitive impairments (Jost et al., 2002). 6. Effects mitochondrial function in several parts of the brain (Streck et al., 2011). 30
- Figure 2: Neurocircuitry overview of reward – Adapted from (Dichter et al., 2012, Treadway and Zald, 2011). The orange lines represent dopamine (DA) projections in the mesolimbic pathway. The yellow lines represent DA projections in the mesocortical pathway. The purple lines represent DA projections in the nigrostriatal pathway. The red line represents  $\gamma$ -aminobutyric acid (GABA) projections and the green lines represent glutamate (GLU) projections. 35
- Figure 3: The modulation of dopamine and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein (with a molecular weight of 32kD) (DARPP-32) - (Wang et al., 2017). Arrows represent activation and blocked lines an inhibition. 39
- Figure 4: Indolamine-2,3-dioxygenase (IDO) and kynurenine pathway altered by cytokine exposure – Adapted from (Haroon et al., 2012). Cytokines

have the ability to activate IDO on peripheral- or brain immune cells. This will lead to the production of kynurenine which will be converted to either kynurenic- or quinolinic acid. Both conversions will have an effect on monoamines, as well as introduce other factors such as oxidative stress. 44

Figure 5: The signalling transduction pathway to c-Fos – (Hudson, 2018). 50

Figure 6: Regulation of the Hypothalamic-Pituitary- Adrenal (HPA) axis – Adapted from Liyanarachchi et al. (2017) and Binder & Nemeroff (2010). 51

Figure 7: Proposed central hub of schizophrenia (SCZ) – Simplified (Steullet et al., 2016). 53

Figure 8: N-acetylcysteine (NAC) pathophysiological targets – (Berk et al., 2013). Transmitter effects: NAC facilitate dopamine (DA) and glutamate (Glu) transmission. Redox modulation: NAC increase glutathione (GSH) which scavenge for reactive oxygen species (ROS) and nitrous oxide (NO). Neurogenesis: Neurogenesis can be directly- or indirectly promoted via NAC administration. Mitochondrial dysfunction: NAC restore mitochondrial dysfunction by altering calcium (Ca<sup>2+</sup>) dynamics. NAC can also reverse mitochondrial toxicity; this will lead to decreased ROS production via altered mitochondria metabolism. Inflammatory response: NAC reduce inflammatory responses by decreasing the cytokines (tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6)). 55

Figure 9: Animal model of psychiatric disorders – Adapted and simplified from Jones et al., (2011). This schematic diagram is a representation of all domains needed for an animal model to be of translational relevance. 58

### CHAPTER 3

Figure 1: Visual representation of the study design. 116

Figure 2 A-D: Conditioned place preference (CPP): **A** (CPP before N-acetylcysteine (NAC) treatment) – Place preference produced by pre-natal lipopolysaccharide (LPS) exposure, post-natal efavirenz (EFV) exposure and a combination of EFV and LPS. Data are expressed as

mean  $\pm$  SEM of 24 animals per group (two-way ANOVA, Tukey's multiple comparisons test). **B** (CPP before NAC treatment) - Place preference produced by pre-natal LPS and saline (SAL) exposure. Data are expressed as mean  $\pm$  SEM of 48 animals per group. \* $p$  < 0.05 vs. SAL (Unpaired student's t-test). **C** (CPP after NAC treatment) - Place preference produced after NAC treatment and previous post-natal EFV exposure, pre-natal LPS exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \* $p$  < 0.05, \*\* $p$  < 0.01 vs. SAL-OO-SAL (three-way ANOVA, Tukey's multiple comparisons test). **D** (CPP after NAC treatment) - Place preference produced by NAC treatment and previous pre-natal exposure to LPS. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \* $p$  < 0.05, \*\* $p$  < 0.01 vs. SAL-SAL (two-way ANOVA, Tukey's multiple comparisons test).

125

Figure 3 A-C: Locomotor activity. **A** (Locomotor activity before N-acetylcysteine (NAC) treatment) - Locomotor activity produced by pre-natal lipopolysaccharide (LPS) exposure, post-natal efavirenz (EFV) exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \*\* $p$  < 0.001, \*\*\* $p$  < 0.0001 vs. saline (SAL) groups (two-way ANOVA, Tukey's multiple comparisons test). **B** (Locomotor activity before NAC treatment) - Locomotor activity induced by pre-natal LPS and SAL exposure. Data are expressed as mean  $\pm$  SEM of 48 animals per group. \*\*\*\* $p$  < 0.0001 vs. SAL (Unpaired student's t-test). **C** (Locomotor activity after NAC treatment) - Locomotor activity produced after NAC treatment and previous pre-natal LPS exposure, post-natal EFV exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \* $p$  < 0.05 vs. LPS-EFV-SAL. \*\* $p$  < 0.01 vs. LPS-OO-NAC. \*\*\*\* $p$  < 0.0001 vs. LPS-EFV-SAL (three-way ANOVA, Tukey's multiple comparisons test).

127

Figure 4 A-C: Average %pre-pulse inhibition (PPI) **A** (Average %PPI before N-acetylcysteine (NAC) treatment): Average %PPI produced by pre-natal lipopolysaccharide (LPS) exposure, post-natal efavirenz (EFV) exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \* $p$  < 0.05 vs. saline-olive oil (SAL-OO) (two-way ANOVA, Tukey's multiple comparisons test). **B** (Average %PPI before NAC treatment): Average %PPI produced by

pre-natal LPS and SAL exposure. Data are expressed as mean  $\pm$  SEM of 48 animals per group. \* $p < 0.05$  vs. SAL (Unpaired student's t-test). **C** (Average %PPI after NAC treatment): Average %PPI produced after NAC treatment and previous pre-natal LPS exposure, post-natal EFV exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test). 129

Figure 5 A-B: Plasma glutathione (GSH): **A** GSH concentration levels in the plasma of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test). **B** Plasma GSH: GSH concentration levels in the plasma of rats after post-natal EFV exposure as well as NAC treatment. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \* $p < 0.05$  vs. EFV-NAC (two-way ANOVA, Tukey's multiple comparisons test). \$ $d > 0.5$ , vs. the olive oil-saline (OO-SAL) group (Cohen's d-value). 131

Figure 6: Plasma corticosterone (CORT): CORT concentration levels in the plasma of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \* $p < 0.05$ , vs. all the other groups (three-way ANOVA, Tukey's multiple comparisons test). # $d > 0.8$ , vs. the saline-olive oil-saline (SAL-OO-SAL) group (Cohen's d-value). 132

Figure 7 A-B: Phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B): **A** Striatal PPP1R1B: PPP1R1B concentration levels in the striatum of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test). # $d > 0.8$ , vs. the saline-olive oil-saline (SAL-OO-SAL) group (Cohen's d-value). **B** Frontal cortical PPP1R1B: PPP1R1B concentration levels in the frontal cortex of rats after exposure pre-natal LPS exposure and/or post-natal EFV exposure as

well as NAC treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test).

133

Figure 8 A-C: Dopamine transporters (DAT): **A** Striatal DAT: DAT concentration levels in the striatum of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \*\* $p < 0.01$  LPS-olive oil (OO)-NAC & LPS-EFV-saline (SAL) vs. all SAL groups, \* $p < 0.05$  LPS-EFV-NAC vs. SAL-OO-SAL, SAL-EFV-SAL, SAL-EFV-NAC (three-way ANOVA, Tukey's multiple comparisons test). **B** Striatal DAT: DAT concentration levels in the striatum of rats after pre-natal exposure to LPS or SAL. Data are expressed as mean  $\pm$  SEM of 48 animals per group. \*\*\*\* $p < 0.0001$  vs. SAL (Unpaired student's t-test). **C** Frontal cortical DAT: DAT concentration levels in the frontal cortex of rats after exposure pre-natal LPS exposure and/or post-natal EFV exposure as well as NAC treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test).

135

Figure 9 A-B: Cerebellar c-Fos **A**: Cerebellar c-Fos: c-Fos concentration levels in the cerebellum of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \*\* $p < 0.01$  vs. LPS-EFV-saline (SAL) & LPS-EFV-NAC, \* $p < 0.05$  vs. LPS-EFV-NAC & SAL groups (three-way ANOVA, Tukey's multiple comparisons test). **B**: Cerebellar c-Fos: c-Fos concentration levels in the cerebellum of rats after exposure pre-natal LPS exposure and/or post-natal EFV exposure. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \*\*\*\* $p < 0.0001$  vs. SAL-olive oil (OO), SAL-EFV and LPS-OO (three-way ANOVA, Tukey's multiple comparisons test).

137

## CHAPTER 4

Figure 1 A-C: A graphical representation of the main bio-behavioural effects observed in this study. A- Sub-acute exposure to efavirenz (EFV), a partial agonist of serotonin 2A receptors (2A or 5-HT<sub>2A</sub>), does not

induce addictive- or psychotic-like bio-behavioural alterations. B- Prenatal exposure to lipopolysaccharide (LPS) induced schizophrenia (SCZ)-like behaviour (hyper-locomotor activity (post-natal day (PND) 55 & 72) and %pre-pulse inhibition (PPI) deficits (PND 55)). One possible mechanism may be ascribed to LPS decreasing striatal dopamine transporters (s.DAT) which resulted in an increase in striatal dopamine (s.DA). C- LPS+EFV exposure: Effects (hyperlocomotor activity and %PPI deficits) induced by LPS became more apparent after EFV exposure (PND 55). This is possibly due to LPS inducing an upregulation of 2A and resulting in an increased response to EFV. LPS+EFV induced an increase in plasma corticosterone (CORT) and decreased s.DAT, all alterations which could contribute towards the SCZ-like behaviour. LPS+EFV also induced an increase in cerebellar c-Fos, which may also be attributed to the upregulation of 2A induced by LPS. This indicated cerebellar involvement in the %PPI deficits and hyper-locomotor activity observed. Behavioural (hyper-locomotor activity and %PPI deficits) and neurochemical (striatal phosphoprotein phosphatase-1 regulatory subunit 1B (s.PPP1R1B)) alterations were attenuated after the N-acetylcysteine (NAC) treatment regimen (PND 72). However, NAC proved to be futile in this study as glutathione (GSH) were reduced when NAC were combined with EFV. Other options were considered such as the effects of LPS+EFV+NAC on plasma CORT levels. The hyper-CORT resulted in an increased dopamine (DA) release within the striatum which could have resulted in the downregulation of the DA<sub>2</sub> receptor (D2).

171

## ADDENDUM B

- |           |   |     |
|-----------|---|-----|
| Figure 1: | Graphical illustration of study design.   | 187 |
| Figure 2: | Visual representation of the conditioned place preference (CPP) apparatus used in our laboratory. | 189 |
| Figure 3: | Visual representation of the pre-pulse inhibition (PPI) apparatus used in our laboratory.         | 192 |

## ADDENDUM C

Figure 1:	A schematic representation of enzyme linked immunosorbent assay (ELISA) methods (Aydin, 2015). A – Direct method, B- Indirect method, C- Sandwich method and D – Competitive method.	217
Figure 2:	Dilution method for working solution.	222
Figure 3:	Standard logistic curve for rat corticosterone (CORT) measured in plasma.	224
Figure 4:	Plate 1- 3 layout for rat corticosterone (CORT) measured in plasma.	225
Figure 5:	Dilution method for working solution.	228
Figure 6:	Standard logistic curve for glutathione (GSH) measured in plasma.	230
Figure 7:	Plate 1- 3 layout for rat glutathione (GSH) measured in plasma.	230
Figure 8:	Dilution method for standard solution.	234
Figure 9:	Standard logistic curve for rat c-Fos measured in the cerebellum.	235
Figure 10:	Plate 1- 3 layout for rat c-Fos measured in the cerebellum.	236
Figure 11:	Dilution method for working solution.	243
Figure 12:	Standard logistic curve for rat dopamine transporters (DAT) measured in the frontal cortex and striatum.	245
Figure 13:	Plate 1- 3 layout for rat dopamine transporters (DAT) measured in the frontal cortex and striatum.	246
Figure 14:	Standard logistic curve for rat phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B) measured in the frontal cortex and striatum.	255
Figure 15:	Test plate and plate 1 layout for rat phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B) measured in the frontal cortex and striatum.	256

## LIST OF TABLES

---

### CHAPTER 1

Table 1:	Post-natal phase – Drug exposure during the six sub-phases of all eight exposure groups.	12
----------	--	----

### CHAPTER 2

Table 1:	Summary of available animal models applicable to psychotic disorders as well as drug abuse and addiction.	59
----------	---	----

### CHAPTER 3

Table 1:	Drug exposure and treatment regimen.	117
----------	--------------------------------------	-----

### CHAPTER 4

Table 1:	A summary of bio-behavioural findings in this study.	168
----------	--	-----

### ADDENDUM B

Table 1:	Sprague-Dawley (SD) female rats – Pre-natal exposure to either saline (SAL) or lipopolysaccharide (LPS).	185
----------	--	-----

Table 2:	Detailed outlay of experimental procedures from November – December 2018. The animals used (November-December) were prenatally exposed to saline.	195
----------	---	-----

Table 3:	Detailed outlay of experimental procedures from March – April 2019. The animals used (March-April) were prenatally exposed to either saline or lipopolysaccharide.	203
----------	--	-----

### ADDENDUM C

Table 1:	Dilution of standard solutions.	233
----------	---------------------------------	-----

Table 2:	Weight of frontal cortical tissue with appropriate phosphate buffered solution (PBS) volume used.	239
----------	---	-----

Table 3:	Weight of striatal tissue with appropriate phosphate buffered solution (PBS) volume used.	240
----------	---	-----

Table 4: Weight of frontal cortical tissue with appropriate phosphate buffered solution (PBS) volume used. 250

Table 5: Weight of striatal tissue with appropriate phosphate buffered solution (PBS) volume used. 251

## **ADDENDUM D**

Table 1: Summary table 259

## LIST OF ABBREVIATIONS

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### #

2A*	Serotonin <sub>2A</sub> receptors
5'-AMP*	5' adenosine monophosphate
5-HT	Serotonin
8-OH-EFV	8-hydroxyefavirenz

### A

AC*	Adenylate cyclase
ACTH	Adrenocorticotropic hormone
AIDS	Acquired immune deficiency syndrome
AMPA*	2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
Amyg*	Amygdala
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AP-1	Activating protein 1
AREC	Animal Research Ethics Committee
ATP*	Adenosine triphosphate
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ARV	Anti-retroviral

### B

BBB	Blood brain barrier
Bcl-2*	B cell lymphoma 2
BDNF*	Brain derived neurotrophic factor

**C**

C/PPI	Conditioned place preference test, followed by a pre-pulse inhibition test
Ca <sup>2+</sup>	Calcium
CaM*	Calmodulin
CaMKII*	Calcium/calmodulin dependant protein kinase II
Camp	Cyclic adenosine monophosphate
CaRE*	Camp response elements
Caud*	Caudate
CER*	Cerebellum
CK*	Creatine kinase
CNS	Central nervous system
CO <sub>2</sub>	Carbon dioxide
CORT	Corticosterone
CPP	Conditioned place preference
CREB*	cAMP-response element binding protein
CRF	Corticotropin-releasing factor
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
Cys*	Cysteine

**D**

D	Dopamine receptors
DA	Dopamine
DARPP-32	DA-and-camp-regulated phosphoprotein (with a molecular weight of 32kd)

DAT Dopamine transporters

DSM-5 The Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders

## E

EDTA Ethylenediaminetetraacetic acid

EFV/E\* Efavirenz

ELISA Enzyme linked immunosorbent assay

EP Eppendorf

Euth\* Euthanasia

## F

FC Frontal cortex

## G

GABA  $\Gamma$ -aminobutyric acid

GD Gestational day

GLP Good Laboratory Practice

GLU/Glu\* Glutamate

Gly\* Glycine

GSH Glutathione

GSSH Oxidised glutathione

## H

HAART Highly active anti-retroviral therapy

HIV Human immunodeficiency virus

HPA Hypothalamic-pituitary-adrenal

HPC/Hipp\* Hippocampus

HRP                    Horseradish Peroxidase

## I

IDO                    Indolamine-2,3-dioxygenase

IEG                    Immediate early gene

IL                      Interleukin

IP                      Intraperitoneally

## J

## K

KO                    Knock-out

## L

LPS                    Lipopolysaccharide

LSD                    Lysergic acid diethylamine

LTP                    Long-term potentiation

## M

M                      Muscarinic

MA                    Methamphetamine

MAO                   Monoamine oxidase

MAPK1/3\*            Mitogen-activated protein kinase 1 and 3

MEK1/2\*             Mitogen activated protein kinase 1 and 2

MIA                    Maternal immune activation

Min                    Minutes

MMP-9                Matrix metalloproteinase 9

MSN Medium spiny neurons

## **N**

Na<sup>+</sup> Sodium

NA Noradrenaline

NAA N-acetylaspartate

NAC N-acetyl cysteine

NAcc Nucleus accumbens

NaCl Sodium chloride

ND\* No drug

NFκB Nuclear factor kappa light chain enhancer of activated B cells

NHREC National Health Research Ethics Council

NMDA N-methyl-D-aspartate glutamine

NMDAR N-methyl-D-aspartate glutamine receptor

NO\* Nitrous oxide

NRF National Research Foundation

NRTI Nucleoside reverse transcriptase inhibitor

NNRTI Non-nucleoside reverse transcriptase inhibitor

NWU North-West University

## **O**

OD Optical density

OO/O\* Olive oil

## **P**

PBS Phosphate buffered solution

PCP	Phencyclidine
PET	Positron emission tomography
PFC	Pre-frontal cortex
PKA	Protein kinase A
PND	Post-natal day
Poly I:C	Polyriboinosinic-polyribocytidylic acid
PP-1	Protein phosphatase 1
PPI	Pre-pulse inhibition
PPP1R1B	Phosphoprotein phosphatase-1 regulatory subunit 1B
Put*	Putamen

## Q

QA	Quinolinic acid
----	-----------------

## R

RasGRF1*	Ras-guanine nucleotide releasing factor
RN	Raphe nuclei
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

## S

s.DAT*	Striatal dopamine transporters
s.PPP1R1B	Striatal Phosphoprotein phosphatase-1 regulatory subunit 1B
SA	South Africa
SAL	Saline
SANAS	South African National Accreditation System

SAVC	South African Veterinary Council
SC	Subcutaneous
SCZ	Schizophrenia
SD	Sprague-Dawley
SEM	Standard error of the mean
SIPD	Substance-induced psychotic disorder
SIR	Social isolation rearing
SN	Substantia nigra
SOP	Standard operating procedure
SRE*	Serum response element
SRF*	Serum response factor

## T

TDO*	Tryptophan 2,3-dioxygenase
Thr34	Threonine 34
TLR	Toll-like receptors
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
TRE*	12-O-Tetradecanoylphorbol-13-acetate response element

## U

## V

VP	Ventral pallidum
VTA	Ventral tegmental area

**W**

WHO World health organization

**X**

**Y**

**Z**

\*Abbreviation only appears in figures and/or tables.

## GLOSSARY

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### **AETHIOLOGY**

The cause, set of causes, or manner of causation of a disease or condition.

### **BIOGENIC AMINES**

There are five established biogenic amines viz. dopamine, serotonin, noradrenaline, adrenaline and histamine.

### **BIO-MARKER**

A naturally occurring molecule, gene, or characteristic by which a particular pathological or physiological process, disease, etc. can be identified.

### **CHEMOKINES**

One of a large group of proteins that act as chemical messengers and were first found attracting white blood cells to areas of inflammation.

### **CO-MORBIDITY**

The presence of one or more additional conditions co-occurring with (that is, concomitant or concurrent with) a primary condition.

### **CYTOKINES**

Any of a number of substances, such as interferon, interleukin, and growth factors, which are secreted by certain cells of the immune system and have an effect on other cells.

### **EPIDEMIC**

A widespread occurrence of an infectious disease in a community at a particular time.

### **EUPHORIA**

A feeling or state of intense excitement and happiness.

### **GENETIC BARRIER**

The number of mutations required to overcome drug-selective pressure.

## **GYRIFICATION INDEX**

A measure of the magnitude of cortical convolutions on the surface of the mammalian brain.

## **HYDROXYLATION**

A chemical process that introduces a hydroxyl group into an organic compound.

## **LATERALIZATION**

Localization of function or activity (as of verbal processes in the brain) on one side of the body in preference to the other.

## **LENTIVIRUS**

Group of retroviruses producing illnesses characterized by a delay in the onset of symptoms after infection.

## **LEUKOTRIENES**

A group of biologically active compounds, originally isolated from leucocytes. They are metabolites of arachidonic acid, containing three conjugated double bonds.

## **LIPOPHILIC**

Tending to combine with or dissolve in lipids or fats.

## **LYMPHOCYTES**

A form of small leucocyte (white blood cell) with a single round nucleus, occurring especially in the lymphatic system.

## **MEDIUM SPINY NEURONS**

A special type of  $\gamma$ -aminobutyric acid inhibitory cell representing 95% of neurons within the striatum, a basal ganglia structure.

## **MORTALITY**

The state of being subject to death.

## **NEUROGENESIS**

The process by which new nerve cells are generated. In neurogenesis, there is active production of new neurons, astrocytes, glia, and other neural lineages from undifferentiated neural progenitor or stem cells.

## **OCCUPATIONAL IMPAIREMENT**

The loss of one's ability to participate in meaningful occupations, which could include activities of daily living, instrumental activities of daily living, rest/sleep, education, play, leisure, work, or social participation

## **PATHOGEN**

A bacterium, virus, or other microorganism that can cause disease.

## **PATHOPHYSIOLOGY**

The disordered physiological processes associated with disease or injury.

## **PHENOTYPIC RESISTANCE**

Achieving resistance without any genetic alteration.

## **PHOSPHORYLATION**

A biochemical process that involves the addition of phosphate to an organic compound.

## **POWER ANALYSIS**

Determine the sample size required to detect an effect of a given size with a given degree of confidence.

## **PREDISPOSITION**

A liability or tendency to suffer from a particular condition, hold a particular attitude, or act in a particular way.

## **PREVALANCE**

The fact or condition of being prevalent; commonness.

**PROGNOSIS**

The likely course of a medical condition.

**PROSTAGLANDINS**

A number of hormone-like substances that participate in a wide range of body functions such as the contraction and relaxation of smooth muscle, the dilation and constriction of blood vessels, control of blood pressure, and modulation of inflammation.

**PSYCHOMIMETIC**

Producing effects (as hallucinations or paranoid delusions) that resemble or are identical with psychotic symptoms.

## CHAPTER 1

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### INTRODUCTION

#### 1.1 Dissertation approach and layout.

This dissertation will be presented in an article format whereas all the key data will be presented in a preliminary (concept) article for possible submission in a peer reviewed scientific journal (Chapter 3). Chapter 3 will also contain all key elements of the study which includes the methods, results and discussion. All supplementary and additional methods, materials, and data will be fully disclosed in the addendums.

The dissertation will adhere to the following format:

- Chapter 1: Introduction

This chapter will discuss the problem statement, study questions, objectives, hypothesis, study layout, expected outcomes and will conclude with the ethical considerations.

- Chapter 2: Literature review
- Chapter 3: Manuscript
- Chapter 4: Concluding remarks, study outcomes, limitations and future recommendations.
- Addendum A: Ethics approval letter
- Addendum B: Pre-natal and post-natal experimental protocols.
- Addendum C: Peripheral- and neurochemical analyses.
- Addendum D: Author guideline: Frontiers in Psychiatry.
- Addendum E: Letters of consent to submit manuscript.

## 1.2 Problem statement

Since the discovery of Human immunodeficiency virus (HIV) in 1959 (Melhuish and Lewthwaite, 2018), and the confirmation of it being the causal agent in acquired immune deficiency syndrome, the calamitous virus has infiltrated the worldwide population and by 2019 the World Health Organization (WHO) reported that an estimated 37.9 million people are infected with HIV (WHO, 2019). Even more concerning is that South Africa (SA) represents 18.7% of this statistic (WHO, 2017<sub>A</sub>). The high prevalence of HIV related deaths (11% (2016)) in SA (UNAIDS, 2019) has implored the need for national intervention as effective treatment for this epidemic are wanting.

Highly active anti-retroviral therapy was set out for HIV in 1996 (Okwundu and Ogunjale, 2018) which comprises several antiretroviral (ARV) drugs within specific treatment classes (Tse et al., 2015). By the end of 2016, 61% of the people living with HIV in SA received ARV therapy (UNAIDS, 2019). This treatment program in SA is regarded as the largest worldwide but it is also largely funded by the government (UNAIDS, 2019). This once again stresses the fact that this epidemic is not only creating a health issue but is also inflicting enormous economic pressure on the government.

The one specific ARV drug that had attracted significant interest is efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor (Ganta et al., 2017). EFV in combination with a nucleoside reverse transcriptase inhibitor is considered as a first-choice treatment for HIV (Meintjes et al., 2017, Arribas, 2003, Kenedi and Goforth, 2011). However, despite EFV being very effective, interest soon waned as clinicians became more aware of neuropsychiatric manifestations that presented promptly after initiating treatment (Marinho et al., 2017). These effects, which vary between mild and moderate severity (Marinho et al., 2017), include depression, vivid dreams, hallucinations, delusions, paranoia, psychosis, manic behaviour (reviewed by (Dalwadi et al., 2018, Kenedi and Goforth, 2011, Gutierrez-Valencia et al., 2009, Gatch et al., 2013)) and cognitive deficits (Apostolova et al., 2015). By reviewing these effects, attention soon shifted to focus on possible complications that may develop, such as a substance-induced psychotic disorder (SIPD) and/or EFV abuse/addiction.

According to the Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders a SIPD is characterized by experiencing either delusions and/or hallucinations (i.e. psychotic symptoms, reviewed by (Garety et al., 2001)) for approximately one month after acute intoxication or sudden withdrawal of a substance (APA, 2013). Addictive substances that possess psychotomimetic properties can aggravate a psychotic response within individuals (with no prior history of a serious mental illness) that will ultimately require emergency treatment (Caton et al., 2005).

Previous case- and review studies describing the neuropsychiatric effects of EFV (de la Garza et al., 2001, Dalwadi et al., 2018, Marinho et al., 2017, Abers et al., 2014, Freudenreich, 2010)

reported that EFV may ultimately induce a psychotic disorder within subjects. It is however noteworthy to mention that these symptoms are much more common in individuals with an existing psychiatric disorder or a history of addiction (reviewed by (Cavalcante et al., 2010)).

Previous literature refers to the abuse of EFV as being used as a recreational drug either independently (Inciardi et al., 2007) or along with other illicit drugs (Larkan et al., 2010) in a mixture known as Nyaope (Mthembi et al., 2019). EFV misuse originated in SA but has spread globally to various metropolitan areas such as Florida (Eban, 2005), Miami (Surratt et al., 2013) and New-York (Davis et al., 2014). Therefore, the abuse of EFV does not only contribute towards an addiction crisis in SA (Grelotti et al., 2014), but also in other parts of the world. It can furthermore undermine the efficacy of anti-HIV therapy as treatment-naïve individuals may develop ARV resistance after prior prolonged exposure to EFV (Grelotti et al., 2013, Larkan et al., 2010).

Earlier this year the Human Sciences Research Council reported that 13.3% of the South African population had used illicit substances during their lifetime (HSRC, 2019). A collective case study done by the University of Pretoria reviewed that Nyaope is readily available in SA and has a particular high prevalence in certain parts of the country (Mahlangu and Geyer, 2018). Nyaope is a very cheap drug, making it easily accessible (Mokwena, 2016), making it extremely problematic considering all the health, social and economic adversities that accompany addiction or abuse of EFV (Larkan et al., 2010).

Literature regarding the mechanisms involved in EFV abuse/addiction and its contributory role towards possible SIPD are limited, as well as research on causal bio-markers. It is therefore of importance to investigate and explore behavioural and neurochemical biomarkers to shed light on the biological basis of this obscure drug-associated effect. Currently a main concern is the ability of EFV to promote oxidative stress (Adjene et al., 2010) by possibly affecting antioxidants in the body such as glutathione (GSH). This may subsequently result in an increase in pro-inflammatory cytokines (O'Mahony et al., 2005) which in turn triggers the release of cortisol (Steensberg et al., 2003) as well as various biogenic amines (Dunn and Wang, 1995) that regulate mood, cognition, sensorimotor gating, anxiety and other neuropsychiatric parameters. In fact, a pre-clinical study determined that EFV's probable reinforcing effects may be due to its interaction with dopamine transporters (DAT), with its psychoactive effects being attributed to affinity and activation of the serotonin (5-HT)<sub>2A</sub> receptor (Gatch et al., 2013). This is not unlike the actions of lysergic acid diethylamine (LSD) (Gatch et al., 2013). Therefore, just like LSD, EFV-mediated 5-HT<sub>2A</sub> stimulation may provoke glutamate (GLU) release in the pre-frontal cortex (PFC) (Ham et al., 2017), resulting in dopamine (DA) release within the nucleus accumbens (Murase et al., 1993), a key brain region implicated in reward processing (reviewed by (Wouterlood et al., 2018)). These neurotransmitters (GLU & DA) regulate a phosphoprotein called DA-and-cyclic adenosine

monophosphate -regulated phosphoprotein (with a molecular weight of 32kD) (DARPP-32) (Foubister, 2002, Svenningsson et al., 2002, Greengard, 2001), also known as phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B) (Wang et al., 2017), which regulates striatal functioning (Dichter et al., 2012). DARPP-32 is of utmost importance for integrating DA and GLU signalling pathways (Albert et al., 2002) and is altered in subjects experiencing a substance abuse problem (Svenningsson et al., 2005) or a psychotic disorder such as SCZ (Albert et al., 2002). An area of interest that has emerged recently is the role of other brain areas, specifically the cerebellum, in addiction and psychotic disorders. c-Fos (a neuronal activity marker (Carbo-Gas et al., 2014) is a bio-marker that has prompted interest in addiction research following the discovery of an association between c-Fos expression and cue-induced preference (Carbo-Gas et al., 2014) as well as elevated c-Fos levels in the cerebellum after exposure to psychomimetic drugs (Näkki et al., 1996).

Although the pathophysiology of addiction and psychotic disorders are influenced by several contributing factors (Ellenbroek et al., 2005, Bolton et al., 2018, Said et al., 2015, Yang et al., 2006, Lakehayli et al., 2015, Hausknecht et al., 2013, Koob et al., 2014, Zavos et al., 2014, Woodward, 2016), this study will consider the association between maternal infection during pregnancy and the development of a psychiatric disorder later in life (specifically addiction and psychotic disorders) (Borçoi et al., 2015, Straley et al., 2017, Boksa, 2010, Fortier et al., 2007).

In 2017, the WHO reported that each year in SA, 930 000 women who are pregnant have an active bacterial infection such as syphilis (WHO, 2017<sub>B</sub>). Numerous pregnant women also present with infections such as pneumonia and tuberculosis (WHO, 2017<sub>C</sub>). Furthermore, one-third of pregnant women in SA are HIV positive (WHO, 2017<sub>C</sub>), and even though mother-to-child transmission can only occur during birth or breastfeeding (Raffe et al., 2017), these patients are in a constant state of immune activation and also experience persistent systemic inflammation (Višković et al., 2018). Pregnancy associated with a comorbid inflammatory state, such as an infection, are enough to alter behaviour and induce neuropathological abnormalities (Patterson, 2009, Zuckerman and Weiner, 2005), therefore contributing to the development of a psychotic or addictive disorder in the off-spring.

Some patients with a psychiatric disorder often present with comorbid substance abuse (a dual-diagnosis) (Temmingh et al., 2014, Lawrie et al., 1995, Regier et al., 1990). It is therefore important to note that psychiatric disorders and addiction have a bidirectional cause (Gregg et al., 2007, Tohen et al., 1998), which implies that either disorder has the ability to increase the susceptibility towards the other disorder (Mueser et al., 1998). Drugs of abuse such as cannabis (Semple et al., 2005), ecstasy (McGuire and Fahy, 1991), amphetamine (Bramness et al., 2012), LSD (Carhart-Harris et al., 2016, Nichols, 2004), and cocaine (Roncero et al., 2013) can induce psychosis. However, patients with a psychiatric disorder, or a prior history of pre-natal stress

(reviewed by (Rodrigues et al., 2011)), frequently present with comorbid substance abuse (Dixon and Haas, 1991).

Moreover, a recent review study implored the academic community to invest in treatment research to alleviate the global psychiatric burden (Phillips et al., 2018), of whatever cause. To our knowledge no literature has been published on a possible pharmacological treatment option for EFV abuse, relapse, possible comorbidities as well as the contributing/exacerbating factors thereof. Considering the proposed role of oxidative stress in EFV-associated neuropsychiatric side effects (Dalwadi et al., 2018), we propose that the antioxidant and GSH precursor N-acetyl cysteine (NAC), may be a suitable candidate for reversing EFV-associated bio-behavioural changes related to addiction as well as SIPD with or without a pre-natal insult.

The aetiology of addiction and psychotic disorders are complex with a vast array of contributing factors including alterations in neurotransmission, inflammation, redox homeostasis and cellular functioning (Dean et al., 2011). NAC possess the ability to influence these factors and play a specific role in the treatment of psychiatric disorders defined by compulsive/impulsive behaviour and altered redox-inflammatory pathways (Sansone and Sansone, 2011, Dean et al., 2011). Previous literature supports this view (Möller et al., 2013, Grant et al., 2007, Dean et al., 2011, Sansone and Sansone, 2011, Chen et al., 2016, Zhou and Kalivas, 2008, Slattery et al., 2015).

Our study will therefore not only focus on the aftermath of EFV abuse, but also explore possible contributing factors as well as developing a possible pharmacological treatment platform. This will be done by investigating rodent behaviour and neurochemical bio-markers after EFV and/or pre-natal inflammation exposure, followed by NAC treatment.

### **1.3 Study questions**

After careful consideration and based on the problem statement above, our study questions are;

1. Will sub-acute EFV exposure induce addictive- and/or psychotic-like behavioural alterations in rats?
2. Will sub-acute EFV exposure induce neurochemical (DAT, c-Fos and PPP1R1B) and peripheral (corticosterone (CORT) and GSH) alterations in rats?
3. Does an early life stressor (pre-natal inflammation induced by lipopolysaccharide (LPS)) alone induce psychotic-like behavioural alterations later in life?
4. Does an early life stressor (pre-natal inflammation induced by LPS) alone induce neurochemical and peripheral alterations later in life?

5. Does an early life stressor (pre-natal inflammation induced by LPS) contribute towards / exacerbate EFV-induced behavioural alterations (if any)?
6. Does an early life stressor (pre-natal inflammation induced by LPS) contribute towards / exacerbate EFV-induced neurochemical and peripheral alterations (if any)?
7. Can a multifunctioning anti-oxidant such as NAC restore bio-behavioural alterations (if any) induced by sub-acute EFV alone or pre-natal inflammation (induced by LPS) alone, as well as the combination thereof?

#### **1.4 Study objectives**

We proposed the following objectives for our study;

1. To investigate whether sub-acute EFV (5 mg/kg) exposure in rats will induce addictive-like behaviour, as found and validated by a previous study performed in our laboratory (Möller et al., 2018). Addictive-like behaviour will be determined using the conditioned place preference (CPP) paradigm.
2. To investigate possible psychotic-like behaviour in rats following sub-acute EFV (5 mg/kg) exposure. This behaviour will be assessed using the pre-pulse inhibition (PPI) test.
3. To investigate possible locomotor alterations induced by EFV (5 mg/kg). This test is measured in the CPP apparatus and can be an indicator to both addictive- and psychotic-like behaviour.
4. To determine whether sub-acute EFV (5 mg/kg) exposure alone will induce alterations in peripheral CORT and GSH levels, cerebellar c-Fos, as well as alterations to DAT and PPP1R1B expression in the striatum and frontal cortex (FC) of rats.
5. To investigate whether an early life stressor (pre-natal inflammation induced by LPS (100 µg/kg)) alone will induce psychotic-like (determined using locomotor activity and the PPI test) behaviour in rats later in life, as previously found and validated in our laboratory (Swanepoel et al., 2018, Harvey et al., 2018).
6. To investigate whether early life pre-natal inflammation (induced by LPS (100 µg/kg)) could contribute towards / exacerbate the above-mentioned bio-behavioural alterations (if any) in rats exposed to sub-acute EFV (5 mg/kg)

7. To establish whether chronic NAC (100 mg/kg) treatment can reverse sub-acute EFV induced bio-behavioural alterations in rats as mentioned above.
8. To establish if chronic NAC (100 mg/kg) treatment could reverse bio-behavioural alterations induced by LPS (pre-natal inflammation) alone in rats.
9. To determine if chronic NAC (100 mg/kg) treatment could reverse bio-behavioural alterations induced by sub-acute EFV (5 mg/kg) in combination with pre-natal inflammation (induced by LPS) in rats.

## 1.5 Hypothesis

Post-natal sub-acute EFV exposure will induce addictive- and psychotic-like behavioural, neurochemical and peripheral alterations in rats later in life. Pre-natal exposure to LPS will induce psychotic-like behavioural, neurochemical and peripheral alterations in rats later in life. The bio-behavioural effects of post-natal EFV exposure will be exacerbated by pre-natal inflammation (as induced by LPS). NAC will reverse the bio-behavioural alterations induced by sub-acute EFV with or without pre-natal inflammation (induced by LPS) exposure, as well as reverse the effects of pre-natal inflammation (induced by LPS) alone.

## 1.6 Project layout

See figure 1A (Pre-natal saline (SAL) exposure cohort) and 1B (Pre-natal LPS exposure cohort) for complete illustration.

Two cohorts were divided into pre-natal- and post-natal phase of which the post-natal phase consisted of six sub-phases. During the pre-natal phase, pregnant Sprague Dawley (SD) rats (n = 24) were divided into two groups; a LPS exposure- (n = 12) (Figure 1B) and SAL exposure group (n = 12) (Figure 1A). The exposure to LPS was on gestational day 15-16, which represents the late first trimester of human pregnancy (Aguilar-Valles and Luheshi, 2011). According to previous pre-clinical studies, this is the maternal exposure period vulnerable to develop psychotic-like behaviour in the offspring (Arsenault et al., 2014, Harvey and Boksa, 2012). The male offspring born from these exposure groups were randomly allocated to eight exposure groups with 12 rats in each group. These eight groups were labelled as follows; 1. SAL-Olive oil (OO)-SAL, 2. SAL-OO-NAC, 3. SAL-EFV-SAL, 4. SAL-EFV-NAC (Figure 1A), 5. LPS-OO-SAL, 6. LPS-OO-NAC, 7. LPS-EFV-SAL, 8. LPS-EFV-NAC (Figure 1B).

The post-natal experiment (started at post-natal day (PND) 48) was divided into six sub-phases, in which the behavioural testing (CPP, PPI & locomotor activity) was done throughout. These six

sub-phases were adapted from a previous pre-clinical addiction study (Barbosa-Méndez et al., 2018) as well as previous work in our laboratory (Möller et al., 2018). This comprehensive study design allows for the investigation of all study questions i.e. development of abuse/addiction and/or psychosis as well as whether NAC treatment will impair the acquisition and reinstatement of a drug-induced place preference (Barbosa-Méndez et al., 2018) and alter drug-induced psychosis. These six sub-phases will be explained in short below, and elaborated on later in the dissertation (Addendum B). See table 1 for a full indication on drug administration and behavioural assessments during this study.

#### Phase 1: Pre-conditioning phase (CPP) (PND 48)

In this phase, the animals didn't receive any exposure to drugs. They were habituated in the CPP apparatus and the most preferred compartment was established.

#### Phase 2: Conditioning phase (CPP) (PND 49-54)

During the conditioning phase, the animals received exposure to either EFV or pharmaceutical grade OO on alternating days from PND 49-54. After this phase, exposure to EFV and OO was stopped.

#### Phase 3: Post-conditioning phase (CPP + PPI + Locomotor activity) (PND 55)

In the post-conditioning phase, the animals didn't receive any exposure to drugs. On this day, the animals underwent CPP testing to establish which compartment is most preferred and locomotor activity was also measured in the CPP apparatus. Thereafter, the animals were subjected to PPI testing.

#### Phase 4: Extinction/Treatment phase (PND 56-69)

During the extinction/treatment phase, the animals received daily exposure to NAC or SAL, depending on the groups they were allocated to.

#### Phase 5: Re-conditioning phase (CPP) (PND 70-71)

In the re-conditioning phase, the animals once again received exposure to either EFV or OO on alternating days.

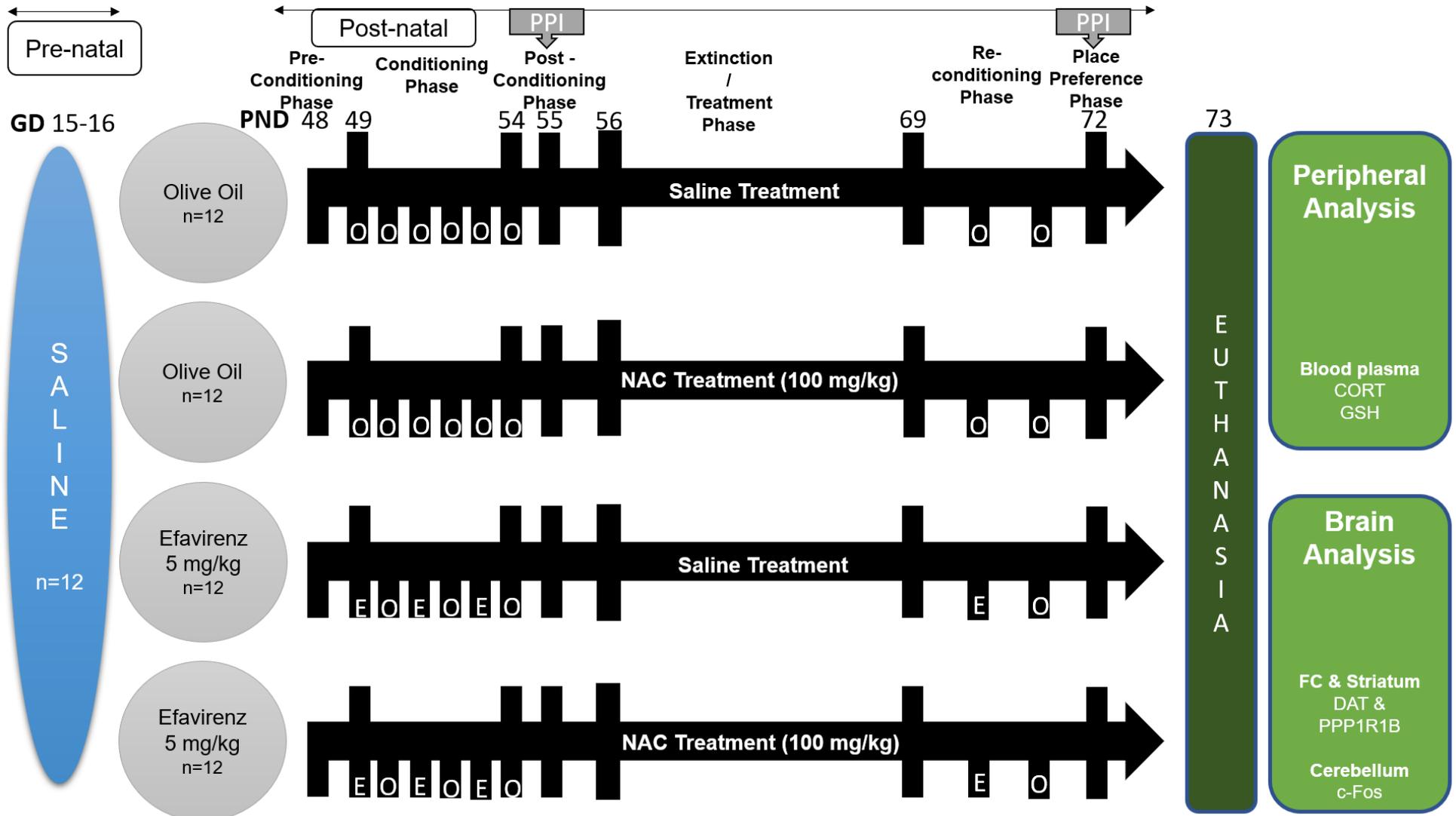
#### Phase 6: Place preference testing phase (CPP + PPI + Locomotor activity) (PND 72)

In the last phase, the animals didn't receive any drug. On this day the animals underwent CPP testing to determine the preferred compartment, to establish whether the treatment effected the reinstatement of the drug-induced place preference as well as locomotor activity. Thereafter, the

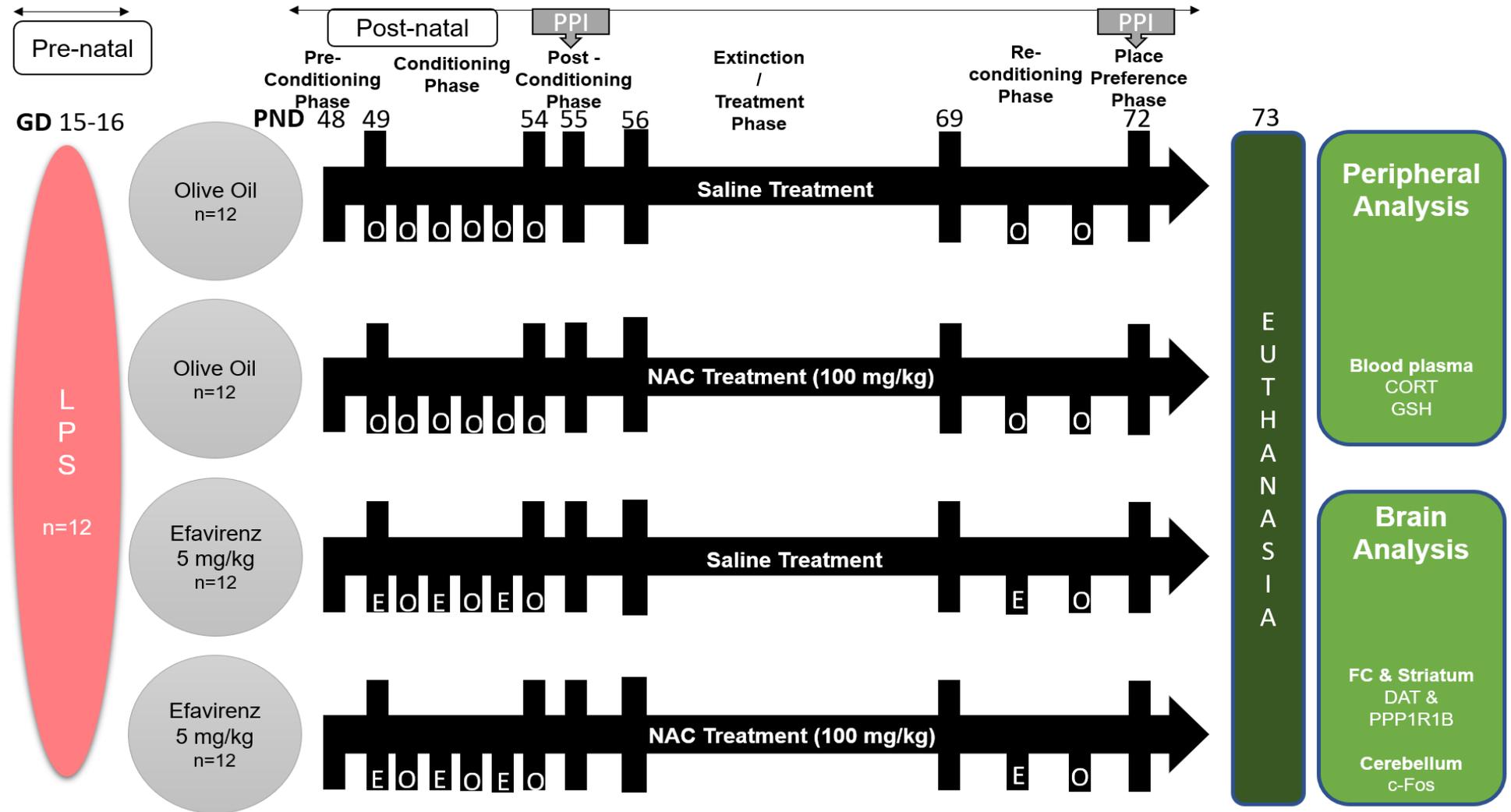
animals were subjected to another PPI test to evaluate whether NAC treatment had an effect on the PPI alterations (if any).

After the six sub-phases, the animals were euthanised on PND 73 after which trunk blood was collected for peripheral analysis and brain tissue for the neuro-chemical analysis.

1A



1B



**Figure 1 A-B:** A visual diagram of the study design as discussed above. A is the pre-natal saline exposure section and B the pre-natal lipopolysaccharide (LPS) exposure section. Abbreviations: E- Efavirenz, O- Pharmaceutical grade olive oil, NAC – N-acetyl cysteine, FC – Frontal cortex, PPI- Pre-pulse inhibition, CCP- Conditioned place preference, DAT- dopamine transporters, PPP1R1B- Phosphoprotein phosphatase-1 regulatory subunit 1B, CORT- Corticosterone, GSH- Glutathione, PND- Post-natal day, GD- Gestational day.

**Table 1:** Post-natal phase – Drug exposure during the six sub-phases of all eight exposure groups.

Post-natal day	Experimental phase	Experimental groups								Behavioural tests
		SAL OO SAL	SAL OO NAC	SAL EFV SAL	SAL EFV NAC	LPS OO SAL	LPS OO NAC	LPS EFV SAL	LPS EFV NAC	
48	Pre-Conditioning Phase	No drug exposure	No drug exposure	No drug exposure	No drug exposure	No drug exposure	No drug exposure	No drug exposure	No drug exposure	CPP
49	Conditioning Phase	OO	OO	EFV	EFV	OO	OO	EFV	EFV	CPP
50	Conditioning Phase	OO	OO	OO	OO	OO	OO	OO	OO	CPP
51	Conditioning Phase	OO	OO	EFV	EFV	OO	OO	EFV	EFV	CPP
52	Conditioning Phase	OO	OO	OO	OO	OO	OO	OO	OO	CPP
53	Conditioning Phase	OO	OO	EFV	EFV	OO	OO	EFV	EFV	CPP
54	Conditioning Phase	OO	OO	OO	OO	OO	OO	OO	OO	CPP
55	Post-Conditioning Phase	No drug exposure	No drug exposure	No drug exposure	No drug exposure	No drug exposure	No drug exposure	No drug exposure	No drug exposure	Complete CPP 1, PPI 1 & Locomotor activity 1
56 – 69	Extinction / Treatment Phase	SAL	NAC	SAL	NAC	SAL	NAC	SAL	NAC	No behavioural tests
70	Re-Conditioning Phase	OO	OO	EFV	EFV	OO	OO	EFV	EFV	CPP

Chapter 1: Introduction

71	Re-Conditioning Phase	OO	CPP							
72	Place Preference Phase	No drug exposure	Complete CPP 2, PPI 2 & Locomotor activity 2							

## 1.7 Expected outcomes

We propose the following outcomes;

- Rats exposed to LPS prenatally and vehicle postnatally will present with a PPI deficit, compared to rats exposed to vehicle pre-and postnatally. They will also present with an increase in locomotor activity but not with any alterations in CPP.
- Rats exposed to LPS prenatally and vehicle postnatally will present with an increase in regional brain (FC & striatum) DAT and cerebellar c-Fos expression as well as a decrease in regional brain (FC & striatum) PPP1R1B, compared to rats exposed to vehicle pre-and postnatally.
- Rats exposed to LPS prenatally and vehicle postnatally will present with an increase in plasma CORT but a decrease in plasma GSH, compared to rats exposed to vehicle pre-and postnatally.
- Rats exposed to vehicle prenatally and EFV postnatally will spend more time in the drug-paired compartment in the CPP, present with PPI deficits and with an increased locomotor activity compared to their control group.
- Rats exposed to vehicle prenatally and EFV postnatally will present with an increase in regional brain (FC & striatum) DAT, PPP1R1B and cerebellar c-Fos expression compared to their control.
- Rats exposed to vehicle prenatally and EFV postnatally will present with an increase in plasma CORT levels, but a decrease in plasma GSH levels, compared to their control.
- Rats exposed to both LPS prenatally and EFV postnatally will present with exacerbated disturbances in all bio-behavioural alterations compared to rats exposed only to LPS prenatally or EFV postnatally.
- NAC treatment will significantly reverse all bio-behavioural alterations in rats exposed to LPS prenatally, rats exposed to EFV postnatally as well as rats exposed to LPS prenatally and EFV postnatally, although the treatment response will be more significant in the latter.

## 1.8 Ethical considerations

The study was submitted to the AnimCare animal research ethics committee (NHREC reg. no. AREC-130913-015) of the North-West University (NWU) and commenced upon ethical approval (Ethics approval number: NWU-00162-18-S5).

All animals used in this study were housed and bred at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform.

Animal research should be conducted accordingly to the concepts of the three R's; Replacement, Refinement, and Reduction (Singh, 2012). In this project, we aimed to design a study that will comply with all the three R's as well as other ethical standards set out by AnimCare.

To implement reduction, a power analysis was done to minimize the number of animals required. Our sample sizes consisted of 12 male SD rats per exposure group, thus, correlating with a similar previous pre-clinical study (Swanepoel et al., 2018). Therefore, the sample sizes are big enough to evaluate the real effects but still small enough not to waste any animals (Fitts, 2011). To implement replacement we thoroughly considered other species as well as other models, finding that rats are still considered to be a better model compared to mice when investigating addictive behaviour (for a full review see (Spanagel, 2017)). Similarly, the LPS model offers important face, construct and predictive validity to the study due to the induction of co-presenting inflammation (see chapter 2 for further discussion). Regarding refinement, we acknowledge that the animals was subjected to a high-stress evoking behavioural test (PPI) as well as to moderate stress (related to multiple intraperitoneal (IP) injections). We aimed to minimize the stress (associated with IP injections), therefore, NAC was administered subcutaneously during the treatment/extinction phase following a method set out by (Swanepoel et al., 2018).

All our testing and handling of the animals were according to the AnimCare standards to ensure that minimal pain and distress are caused. In our study, we only used male offspring as it is known that the female reproductive cycle can significantly influence each stage of addiction (for a full review on each stage see (Becker and Koob, 2016)) as well as alter PPI test results (Swerdlow et al., 1997). The female dams used in this study along with the female offspring born from the pregnant dams were euthanised by carbon dioxide (CO<sub>2</sub>) overdose as set out by the standard operating procedure (Euthanasia of Rodents: SOP-Viv-Anim 1) of the Vivarium.

The estimated animal experience category for this study is a 4 (very severe) due to numerous factors such as;

- The stress incurred when pregnant SD rats are injected with LPS.
- The stress associated with drug administration (EFV) via an IP injection.

- The high-stress evoking behavioural test (PPI), as well as stress caused by restraining of the animal within the cylinder of the apparatus for more than 30 min.

The rats were monitored daily during this study for any distress or discomfort via a study-specific NWU Vivarium monitoring sheet. Additional monitoring was applied two hours after the rats underwent PPI testing to ensure the absence of excessive distress or behavioural abnormalities. When it was found that a rat is suffering (a score between 10 & 15 – as indicated on approved monitoring sheets), the animal was considered for humane euthanasia and only if a score above 15 was obtained. In this case, immediate euthanasia (CO<sub>2</sub> overdose) was applied by a qualified, experienced animal technician. During this study, no animal had excessive distress or any abnormal behaviour that required intervention from the researcher or the animal technician.

Plastic (polyvinyl chloride) tubes with corncob bedding were placed inside the standard solid floor cages for nesting, resting and environmental enrichment purposes. The researcher handled the animals on a regular basis to allow them to become familiar with human touch and to minimize any additional stress during behavioural testing as well as drug and vehicle injections. The monitoring reports of this study were submitted every six months after ethical approval had been obtained. Any adverse events, amendments or incidences were reported immediately (within 24 hours of incidence or adverse event) to the AnimCare committee. It is however noteworthy to mention that no adverse events or incidences were reported during this study.

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## CHAPTER 2

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### LITERATURE REVIEW

#### **1.1 Human immunodeficiency virus (HIV), Acquired Immune Deficiency Syndrome (AIDS) & Highly active anti-retroviral therapy (HAART).**

HIV is a blood borne pathogen that was discovered 60 years ago in an adult male in the Democratic Republic of Congo (Melhuish and Lewthwaite, 2018). This lentivirus originated as a result of cross-species (primate to human) transmission via hunting practices in central Africa (Melhuish and Lewthwaite, 2018). Constant HIV replication on elevated levels can cause severe immune suppression, as the virus targets the immune cells such as the CD4 lymphocytes (Ramana et al., 2014, Melhuish and Lewthwaite, 2018). This ultimately progresses to AIDS which is a chronic destructive disease (Kontomanolis et al., 2017) defined by the high probability of opportunistic infections (Melhuish and Lewthwaite, 2018).

HIV can be divided into two subtypes, viz. HIV-1 and HIV-2, both originating from different primates, and differ substantially in prognoses and treatment (Biswas et al., 2018). While HIV-1 is a more aggressive virus, patients infected with HIV-2 have a much more promising prognosis, with only a 30% chance to develop AIDS and a higher life-expectancy (Biswas et al., 2018, Melhuish and Lewthwaite, 2018).

According to the most recent data (2019) of the World Health Organization, 37.9 million people worldwide are infected with HIV (WHO, 2019). Disturbingly, 7.1 million of those people reside in South Africa (WHO, 2017), which accentuates the urgency of a nation-wide intervention. Despite HIV/AIDS being a manageable disease (Colvin, 2011), the global number of new infections increased by 1.7 million in 2018 (UNAIDS, 2019), thereby emphasizing the need for a worldwide intervention as well.

HAART introduced combination therapies with multiple antiretroviral (ARV) drugs and focused on HIV-1 (Ramana et al., 2014). HAART inhibits HIV-1 replication which will reduce viral load as well as the other co-morbidities associated with HIV/AIDS in the long term (Ramana et al., 2014). The current HAART for HIV consists of several classes, such as; nucleoside reverse transcriptase inhibitor (NRTI) (lamivudine, zidovudine etc.), non-nucleoside reverse transcriptase inhibitor (NNRTI) (efavirenz, nevirapine, etravirine etc.) and nucleotide reverse transcriptase inhibitor (e.g. tenofovir) (Ramana et al., 2014, Tse et al., 2015). More classes include; protease inhibitor (e.g. indinavir, atazanavir), entry inhibitor (e.g. maraviroc), fusion inhibitor (e.g. enfuvirtide), integrase inhibitors (e.g. raltegravir, dolutegravir) and co-receptor antagonists (Tse et al., 2015, Ramana et al., 2014). Since the introduction of HAART in the 1990's the mortality rate has significantly decreased with an increase in life expectancy (Cardoso et al., 2013, Luma et al., 2012). However,

as stated above the number of new HIV infections did not decrease (UNAIDS, 2019) and may be due to factors such as non-adherence to the regimen (Robison et al., 2008), or early discontinuation due to adverse effects (Subbaraman et al., 2007).

## 1.2 Efavirenz (EFV)

Since the approval of EFV [(S)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one] in 1998 (Apostolova et al., 2017), it has been a drug of choice in HIV therapy (Best and Goicoechea, 2008) due to its extreme efficacy and favourable pharmacokinetic profile (Apostolova et al., 2015). It is administered in a 600 mg dose along with two NRTI's (usually tenofovir and emtricitabine or lamivudine) to treatment naïve patients (Meintjes et al., 2017).

EFV has a distinct pharmacological profile. It is 99% bound to plasma proteins (Boffito et al., 2003, Wynn et al., 2002) which ensures a high plasma/intracellular concentration ratio (Apostolova et al., 2017). EFV is a lipophilic drug (Wynn et al., 2002) allowing it to reach the central nervous system (CNS) by crossing the blood brain barrier (BBB) (Best et al., 2011, Tashima et al., 1999). Although this is advantageous in treating central HIV-1 infection, it also is associated with unwanted CNS adverse effects that patients often experience while using the drug (Best et al., 2011, Tashima et al., 1999).

EFV is metabolized by the liver to produce inactive metabolites (Ward et al., 2003), primarily via the cytochrome P450 (CYP) system (Smith et al., 2001). These metabolites (8-hydroxyefavirenz (8-OH-EFV) and 7-hydroxyefavirenz) are formed through hydroxylation via the CYP 2B6 isoform (Apostolova et al., 2017). Even though inter-individual enzyme variability may cause inter-patient differences in the biotransformation of EFV (Smith et al., 2001), the phenolic metabolite 8-OH-EFV, a pro-oxidant compound (Grilo et al., 2017, Harjivan et al., 2014), appears to be involved in several mechanisms associated with CNS toxicity (Grilo et al., 2017). Previous literature reported an association of 8-OH-EFV with EFV related mood fluctuations (Grilo et al., 2016), neurotoxicity resulting from 8-OH-EFV effects on astrocyte metabolism (Brandmann et al., 2013), and redox profile changes as observed in the liver and hippocampus (HPC) (Grilo et al., 2017).

The CNS effects vary in severity, and although for most patients they tend to disappear after the first month of therapy (Gutierrez-Valencia et al., 2009, Kenedi and Goforth, 2011), they could persist for up to a year (Leutscher et al., 2013). As reviewed by Apostolova *et al.*, (2017) 60-90% of patients using EFV will experience neuropsychiatric effects such as sleep disturbances (e.g. insomnia and night terrors), euphoria, nervousness and irritability. The authors furthermore elaborated on the 2% of patients that experience severe neurological symptoms i.e. depression, aggressive behaviour, anxiety, psychosis (similar to a reaction gained from lysergic acid diethylamine (LSD) use (Arendt et al., 2007)), hallucinations, paranoia, and delusions (Apostolova et al., 2017, Gatch et al., 2013). A review study reported on numerous case reports where

individuals using EFV experience manic and psychotic episodes so severe, some needed hospitalization and neuroleptic treatment (Kenedi and Goforth, 2011). The authors furthermore highlighted that severe EFV-induced adverse effects resolved upon discontinuation.

A review done by Dalwadi *et al.*, (2018) reported that neuropsychiatric adverse events were not only found in patients using EFV, but also in patients using ritonavir, zidovudine, emtricitabine, tenofovir, abacavir as well as nevirapine as part of their ARV therapy. These findings are in line with multiple individual studies that found traces of zidovudine (Khine *et al.*, 2015), nevirapine (Mthembi *et al.*, 2018) and ritonavir (Grelotti *et al.*, 2014) in a combination with other addictive substances to make up designer drugs. This warrants more attention on ARV therapy and their resulting effects on mental health.

Despite the CNS effects mentioned above, EFV is still considered an efficient and potent (reviewed by (Fortin and Joly, 2004)) NNRTI. However, EFV possesses a low genetic barrier and is susceptible for phenotypic resistance as mutations can occur on the K103N reverse transcriptase gene after persistent exposure to sub-therapeutic drug levels (reviewed by (Marzolini *et al.*, 2001)). Subsequently more light was shed on the severity as well as implications of the above-mentioned, with previous literature reporting that NNRTI drug-resistance is most common in the majority of ARV- experienced patients as well as being the most frequent type transmitted to ARV- naïve patients (reviewed by (Margot *et al.*, 2009)). This then causes a significant problem in the HAART regime, as the therapy becomes suboptimal leading to failure, thereby creating an enormous challenge for controlling the AIDS epidemic (Luo *et al.*, 2019).

### 1.2.1 EFV abuse and mechanisms of EFV-related neuropsychiatric effects.

EFV abuse originated 19 years ago when traces of EFV was found in a new South African designer drug known as “Nyaope” or “Whoonga” (Mokwena, 2015, Grelotti *et al.*, 2014). This drug is a blend of various street drugs such as opioids, acetaminophen, amphetamine, and dextromethorphan (Khine *et al.*, 2015, Mthembi *et al.*, 2018), also containing ARV's (e.g. EFV) (Khine and Mokwena, 2016, Mthembi *et al.*, 2018), laundry detergent and rat poison (Hull, 2010). This mixture is powdered (Khine *et al.*, 2015), mixed with marijuana and then smoked or injected (Hull, 2010).

There are ongoing debates over EFV abuse, more specifically pertaining to the mechanisms responsible for its psychiatric- and psychedelic effects. Cavalcante *et al.*, (2010) proclaimed that the neurochemical pathways influenced by EFV as well as the mechanisms where by psychiatric effects are provoked are unclear and warrant further investigation. According to some scientists, it is EFV alone that is responsible for the above manifestations (Gatch *et al.*, 2013, Gutierrez-Valencia *et al.*, 2009) while others argue that it is due to an interaction between EFV and the other

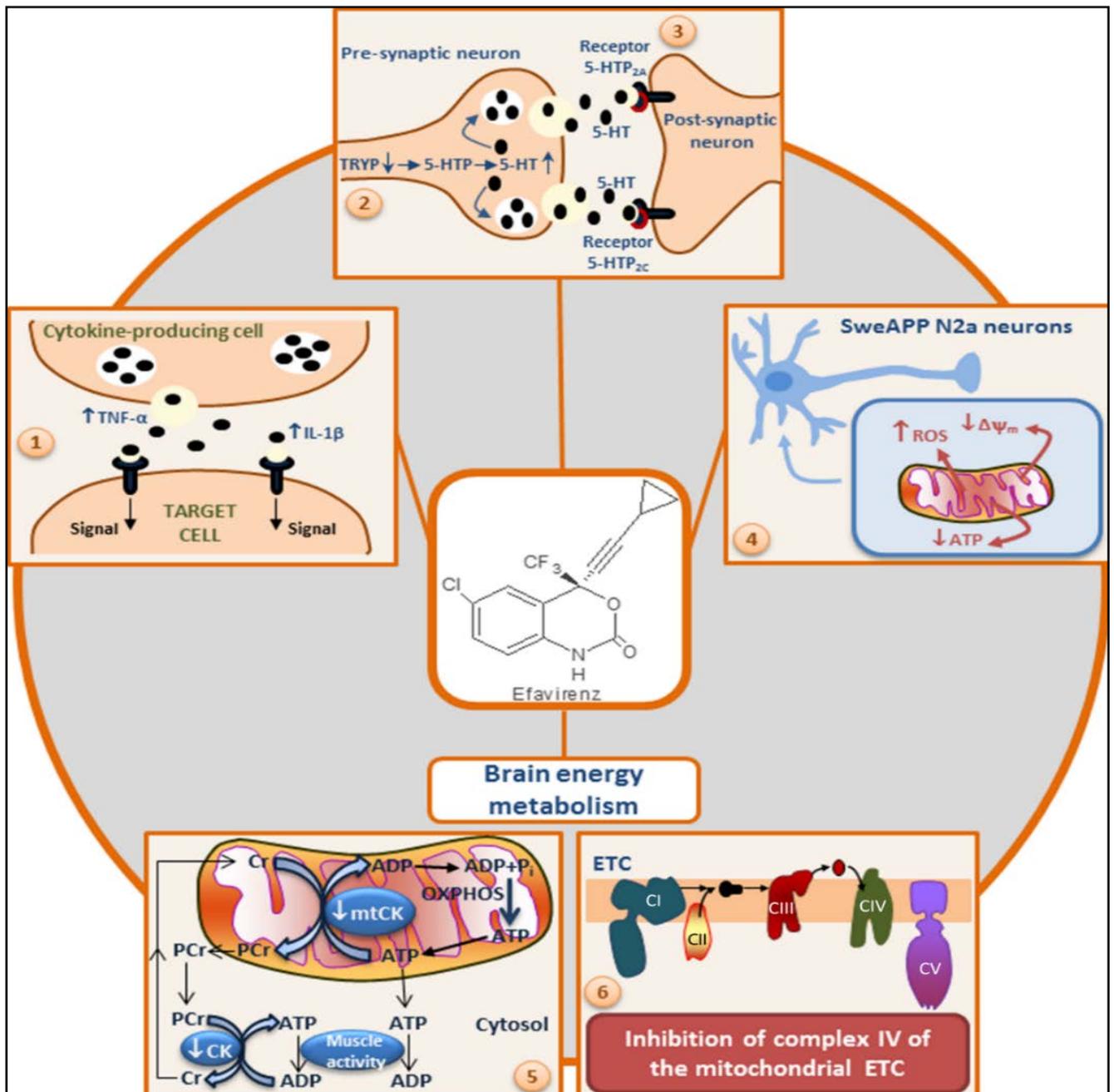
constituents of the “Nyaope”-cocktail (see (Antoniou and Tseng, 2002) for a review on the interaction between ARV’s and recreational drugs).

Gatch et al., (2013) pointed out that these effects are probably attributed to the lysergic LSD-like properties of EFV. Similar to LSD, EFV has an affinity (relative low) for the serotonin (5-HT)<sub>2A</sub> receptor, acting as a partial agonist (Gatch et al., 2013). These authors also found that mice exposed to EFV showed a head twitch response, a behavioural expression of hallucinogenic potential in humans. This response was confirmed to involve activation of 5-HT<sub>2A</sub> receptors as it was absent in 5-HT<sub>2A</sub> knock-out (KO) mice (Gatch et al., 2013). Other identified targets for EFV included 5-HT<sub>2C</sub> and  $\gamma$ -aminobutyric acid (GABA)<sub>A</sub> receptors, 5-HT- and dopamine transporters (DAT), and the vesicular monoamine transporter<sub>2</sub> (Gatch et al., 2013).

In contrast with the above-mention study, Dalwadi et al., (2016) reported that EFV is not a weak agonist of 5-HT<sub>2A</sub> but rather an antagonist of the 5-HT<sub>2A</sub> receptor and competes with LSD to bind to the 5-HT<sub>2A</sub> receptor. This study furthermore stated that EFV acts as an inverse agonist on the 5-HT<sub>6</sub> receptor, 5-HT<sub>3A</sub> ionotropic receptor blocker, an antagonist at muscarinic (M) M<sub>1</sub> and M<sub>3</sub> receptors, and a modest inhibitor of monoamine oxidase (MAO)-A (Dalwadi et al., 2016). Dalwadi et al., (2016) suggested that the hallucinations do not occur due to the partial agonism on the 5-HT<sub>2A</sub> as reported by Gatch et al., (2013) but rather due to antagonism of muscarinic M<sub>1</sub> and M<sub>3</sub> receptors.

The most recent pre-clinical study established that EFV alone possesses dose-dependent addictive-like properties (Möller et al., 2018). These authors found that sub-acute and sub-chronic exposure to EFV (5 mg/kg) induced drug seeking behaviour that is similar in severity to methamphetamine (MA) and delta9-tetrahydrocannabinol, two known drugs of abuse. The authors suggest that these behavioural actions of EFV could be ascribed to elevated levels of cortico-striatal dopamine (DA) and 5-HT as well as striatal noradrenaline (NA).

Numerous articles have proposed other mechanisms for EFV’s adverse CNS effects (O’Mahony et al., 2005, Cavalcante et al., 2010, Gatch et al., 2013, Adjene et al., 2010, Brown et al., 2014, Streck et al., 2008, Streck et al., 2011). See figure 1 for a summary of the proposed mechanisms. A recent review by Dalwadi and colleagues synthesized evidence from the literature to propose the most likely mechanism. They propose that EFV induces a complex array of effects that ultimately lead to redox-inflammatory disturbances that in turn initiate its neuropsychiatric and addictive like properties (Dalwadi et al., 2018). This provides a useful guideline for ideas on how best to treat EFV-induced neuropsychiatric side effects by pharmacological means.



**Figure 1:** Proposed mechanism of efavirenz (EFV) to induce central nervous system adverse effects (Apostolova et al., 2015). 1. EFV causes an increase in interleukin (IL)-1 $\beta$  and tumour necrosis factor (TNF)- $\alpha$  (pro-inflammatory cytokines) (O'Mahony et al., 2005). 2. A correlation between an increase in serotonin (5-HT) levels and a decrease in the activity of tryptophan 2,3-dioxygenase (TDO) (Cavalcante et al., 2010). 3. EFV has an affinity for the 5-HT receptors and shows partial agonist activity (Gatch et al., 2013). 4. EFV promotes oxidative stress (Adjene et al., 2010, Brown et al., 2014). 5. Creatine kinase (CK) inhibition in cerebellum, cortex, striatum, and hippocampus (Streck et al., 2008) which could result in cognitive impairments (Jost et al., 2002). 6. Effects mitochondrial function in several parts of the brain (Streck et al., 2011).

### **1.3 Substance-induced psychotic disorder (SIPD), schizophrenia (SCZ) drug abuse and addiction**

#### 1.3.1 SIPD

The Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) describes a SIPD as a state where a patient experiences hallucinations and/or delusions after acute intake or withdrawal of a psychomimetic agent (e.g. hallucinogens, stimulants, alcohol, cannabis & cocaine) (APA, 2013). These symptoms will be evident in patients for a considerable time period (more or less a month or for as long as the substance has been used) and promote extreme occupational- and social impairment which requires immediate medical attention (APA, 2013).

Recent studies have highlighted that up to 46% of SIPD patients are at risk of developing SCZ within three years after the first episode (reviewed by (O'Connell et al., 2019, Alderson et al., 2017)). Despite the development and course of a SIPD being characterized by the type of substance used (APA, 2013), scientists claim a direct association between the underlying mechanisms present in SCZ patients and those experiencing psychotic episodes induced by drugs of abuse (Alderson et al., 2017, Paparelli et al., 2011)

All drugs of abuse influence the mesolimbic DA-ergic pathway which are evident in the development of addiction, psychotic symptoms and SCZ (Fiorentini et al., 2011). A review by Paparelli and colleagues elaborated on neurochemical (DA, 5-HT and glutamate (GLU)) alterations induced by some psychomimetic drugs (LSD, amphetamine, cannabis & phencyclidine (PCP)) that correlated with the alterations found in SCZ patients (Paparelli et al., 2011). Other literature has focussed attention on a mutual genetic mutation, where individuals with an inherent vulnerability towards a chronic psychotic disorder are more at risk to develop a psychotic episode upon intoxication (Bramness et al., 2012, Ikeda et al., 2013). The latter is in line with previous literature that showed low dosages of psychostimulants induce psychotogenic effects within SCZ patients but not in healthy subjects (reviewed by (Lieberman et al., 1987)).

#### 1.3.1.1 SCZ

SCZ is a serious chronic psychiatric disorder which burdens patients with an immense disability to function within society (Gomes et al., 2019). This disorder currently affects 23 million people globally (WHO, 2018), thereby contributing significantly towards the Global Burden of Disease (Whiteford et al., 2013). Despite extensive research into this disorder, a specific aetiology has not yet been established, possibly due to a vast array of risk- and contributing factors (explained later in this section). DSM-5 defines SCZ as a combination of positive-, negative- and cognitive symptoms upon which diagnosis is based (APA, 2013).

### *Positive symptoms*

A primary positive symptom experienced by patients with SCZ is hallucinations, which is an involuntary false perception with an adequate realistic quality that a person experiences without an external stimulus (Goldsworthy and Whitaker, 2015, Fletcher and Frith, 2009). Hallucinations demonstrate a sensory connection, meaning hallucinations can be tactile, visual, olfactory, gustatory or auditory (Momeni and Raghibdoust, 2012). On that note, however, visual- and auditory hallucinations are most common (Goldsworthy and Whitaker, 2015). Patients also experience delusions which are bizarre beliefs even though persuasive evidence fails to convince otherwise (Fletcher and Frith, 2009, Naasan, 2016). Disorganized thoughts or thought disorder is another positive symptom manifestation (Migdalska-Richards and Mill, 2019). Thought disorder can be defined as an intricate disorder where patients experience impairments within social cognition and language processing as well as thought disturbances (Thomas and Fraser, 1994).

### *Negative symptoms*

The negative symptoms of SCZ are mostly difficult to evaluate, although health practitioners associate them with a decline in normal functioning that is severely disabling to the patient (Buckley and Stahl, 2007). These symptoms can be characterized by five different domains, viz. dysfunction of motivation (avolition), -communication (alogia), -socialisation (asociality), -affect (blunted affect or depressed mood) and -capacity for pleasure (anhedonia) (Buckley and Stahl, 2007).

### *Cognitive symptoms*

Renowned researchers in the field of SCZ (Kraepelin and Bleuler) have always placed significant attention on the fact that SCZ is primarily a cognitive illness and that it should be approached as one (reviewed by (Reddy and Mythri, 2016)). Cognitive symptoms usually precede the manifestation of an acute episode and worsen progressively during the course of the illness (Lewis, 2004, Tripathi et al., 2018). Most startling is that 98% of SCZ patients experience cognitive deficits (Keefe et al., 2005), while 66% of the presenting symptoms are extremely severe and disabling (Lewis, 2004). According to Tripathi et al., (2018) the primary causal factor for cognitive deficits can be attributed to abnormal neurodevelopment. As will be discussed later (Section 1.5), the foundation for these abnormalities begins during early life, i.e. pre-natal or early post-natal.

Cognitive symptoms are divided into seven functional domains which are (Buchanan et al., 2011);

1. Social cognition
2. Verbal learning and memory
3. Processing speed

4. Visual learning and memory
5. Problem-solving
6. Attention
7. Working memory

### 1.3.2 Drug abuse and addiction

Drug abuse and addiction are two concepts that reside close to each other yet differ significantly in meaning. The distinction between them is of great importance as studies and pharmacological approaches to treatment vary considerably (Erickson, 1995).

Abuse is a conscious decision made by the user that eventually presents with pronounced social problems (Erickson, 1995). Addiction is a chronic convoluted disorder where the victim is severely affected at psychological and biological levels (Yadid et al., 2018, Zilverstand et al., 2018). Addiction is characterised by a three-stage relapsing cycle which consists of: intoxication/binge, withdrawal and craving/preoccupation (Uhl et al., 2019, Zilverstand et al., 2018). This cycle is the driving force behind compulsive drug use and drug-seeking (developed after recreational drug use) regardless of any detrimental consequences (Nestler and Lüscher, 2019, Zilverstand et al., 2018).

Factors such as conditioning effects, stress, environmental factors, genetic variables (Koob et al., 2014), and early life events (Ellenbroek et al., 2005) contribute towards the vulnerability of an individual to enter this cycle. Numerous pre-clinical studies in animals have concluded that early life events such as limited bedding and nesting (Bolton et al., 2018) as well as pre-natal stress (Yang et al., 2006, Hausknecht et al., 2013), including pre-natal inflammation (Borçoi et al., 2015, Straley et al., 2017), contribute towards this vulnerability. Similarly, clinical studies (including case-control- and retrospective cohort studies) have also found that early life events such as child abuse (Marcenko et al., 2000), childhood sexual abuse (Wilsnack et al., 1997), childhood neglect and household dysfunction (Dube et al., 2003) as well as pre-natal inflammation (see (Scola and Duong, 2017) for a review) influence the development of addiction. These early life adversities underlie the neurodevelopmental basis of the disorder, as discussed in section 1.5

## 1.4 Neuroanatomy and neurochemistry

When looking at the aetiology of drug addiction along with the neuroanatomy and -chemistry involved in both SCZ and abuse/addiction, a lot of factors needs to be considered. Neurotransmitters such as DA, 5-HT, GLU, GABA and NA play a significant role and should be kept in mind along with other factors such as early and late life stressors. For the purpose of this study we will only focus on DA, 5-HT and the impact of early life stress. Despite several brain regions implicated in SCZ and abuse/addiction we primarily focussed on the frontal cortex (FC)

and striatum, as previous literature emphasized their significance within each respective disorder (Weiss, 2005, Liddle and Pantelis, 2003). Furthermore, we aimed to investigate beyond the normal regions implicated and therefore also investigate the cerebellum (discussed in section 1.7).

#### 1.4.1 DA

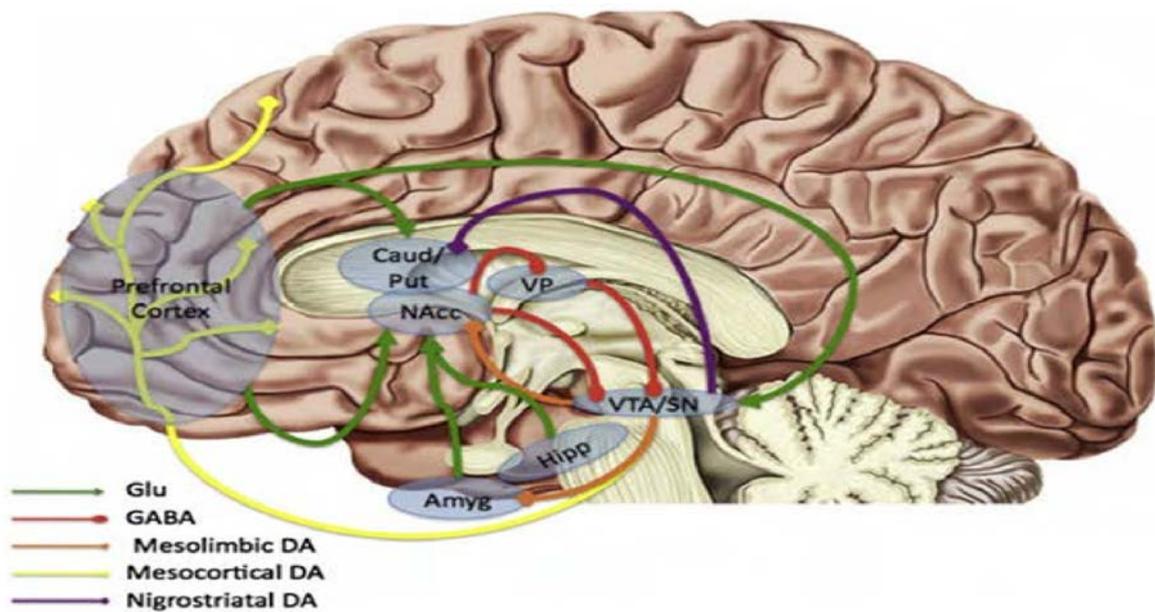
##### SCZ

DA or 3-hydroxytyramine is one of the first inhibitory neurotransmitters implicated in the aetiology of SCZ. This catecholamine is produced in the ventral tegmental area (VTA) and substantia nigra (SN) from where it sends projections to selected brain regions via the mesocortical, mesolimbic, tuberoinfundibular and nigrostriatal pathway (reviewed by (Brisch et al., 2014, Beaulieu and Gainetdinov, 2011)) (Figure 2). DA is involved in numerous vital functions and the dysfunction thereof is evident in numerous disorders (e.g. SCZ, addiction, Parkinson's disease, Tourette's syndrome, bipolar disorder and depression) (Beaulieu and Gainetdinov, 2011).

The current DA hypothesis stipulates that the positive-, negative- and cognitive symptoms are directly related to DA alterations in the mesolimbic- and mesocortical pathways (Walter et al., 2009). The positive symptoms derive from a hyperactive mesolimbic DA projection, which results in a hyperstimulation of DA type 2 receptors (D<sub>2</sub>) (Walter et al., 2009). The negative- and cognitive symptoms are due to a hypoactive mesocortical DA projection to the prefrontal cortex (PFC), leading to the hypostimulation of D<sub>1</sub> receptors (Walter et al., 2009). This hypothesis is supported by evidence such as psychoactive effects induced by dopaminergic substances and also D<sub>2</sub> blockers being clinically accepted as a treatment for psychosis (reviewed by (Beaulieu and Gainetdinov, 2011)).

##### *Drug abuse & addiction*

Although all drugs of abuse have one thing in common, viz. to produce euphoria and a feeling of reward (Gutman, 2006), they have distinct pharmacological profiles and generate different behavioural effects (Wise, 1996). The latter are induced by influencing the mesocorticolimbic DA system (see figure 2) (Gutman, 2006, Wise, 1996).



**Figure 2:** Neurocircuitry overview of reward – Adapted from (Dichter et al., 2012, Treadway and Zald, 2011). The orange lines represent dopamine (DA) projections in the mesolimbic pathway. The yellow lines represent DA projections in the mesocortical pathway. The purple lines represent DA projections in the nigrostriatal pathway. The red line represents  $\gamma$ -aminobutyric acid (GABA) projections and the green lines represent glutamate (GLU) projections. Abbreviations: Amyg – Amygdala, Hipp – Hippocampus, VP – Ventral pallidum, VTA – Ventral tegmental area, SN - Substantia nigra, NAcc – Nucleus accumbens, Caud– Caudate, Put - Putamen.

The origin of the mesocorticolimbic DA pathway is the VTA or SN (Weiss, 2005) which are responsible for DA projections towards the mesolimbic-, mesocortical- and nigrostriatal DA pathways (Dichter et al., 2012). The mesolimbic pathway consists of the nucleus accumbens (NAcc), amygdala and the HPC. The mesocortical DA pathway consists of the PFC while the nigrostriatal DA pathway involves the caudate and putamen (Dichter et al., 2012). After receiving said DA projections, the HPC, amygdala and PFC send excitatory GLU-ergic projections to the NAcc (Dichter et al., 2012). The PFC can also project excitatory GLU-ergic projections to the VTA and/or SN as well as the caudate and putamen (Dichter et al., 2012). After receiving GLU projections, the NAcc sends an inhibitory GABA-ergic projection to the ventral pallidum (VP), which acts to inhibit the inhibitory effect of the VP on the VTA and SN (Dichter et al., 2012). This process will then facilitate a burst of DA firing, leading to a feeling of reward or euphoria (Dichter et al., 2012).

The release of DA within the NAcc, as induced by drugs of abuse, and the subsequent conditioned responses triggered in both addicted and non-addicted individuals is a clear indication that a simple change in DA alone is not responsible for the entire addiction phenotype (Volkow and Morales, 2015). The transitioning is therefore associated with the switch from NAcc involvement (rewarding properties) to the dorsal striatum (involved in habit formation) (Everitt and Robbins, 2013). Addiction is a slow-developing disorder, although certain factors (type of abusive

substance, exposure pattern, genetics and developmental stage) can influence the pace of transition from reward to addiction (Robins and Przybeck, 1985, Kessler et al., 2007).

DA has an effect on numerous physiological functions via these pathways, i.e. hormone secretion, movement coordination and emotional and motivated behaviours (Beaulieu and Gainetdinov, 2011, Hornykiewicz, 1966), where the dysfunction thereof is implicated in the development as well as persistence of addiction (Franken et al., 2005). This is due to repeated exposure to drugs of abuse resulting in adaptive changes on a cellular and molecular level within the mesolimbic pathway. As a result, motivational-, contextual- and emotional behaviours are influenced (Steketee and Kalivas, 2011, Nestler and Carlezon, 2006).

Once DA is produced and projected to certain brain regions (e.g. NAcc and ventral striatum, respectively), it will interact with the D receptors located on medium spiny neurons (MSN) (Cooper et al., 2017). D receptors are divided into two main groups, viz. DA<sub>1</sub>-like receptors (D<sub>1</sub> & D<sub>5</sub>) and D<sub>2</sub>-like receptors (D<sub>2</sub>, D<sub>3</sub>, D<sub>4</sub>) (reviewed by (Baik, 2013)).

Both the D<sub>1</sub> and D<sub>2</sub> are involved in the reinforcing properties of drugs of abuse (Le Foll et al., 2009) as well as the locomotor effects (Kalivas and Stewart, 1991, Beaulieu and Gainetdinov, 2011). Previous pre-clinical- and clinical studies have proven DA involvement in alcohol, nicotine, opiate and psychostimulant abuse (for a comprehensive review see (Le Foll et al., 2009)).

According to the review by Volkow et al., (2011), all drugs of abuse (that will ultimately lead to substance addiction) significantly increase DA within the ventral striatum (NAcc is located in the ventral striatum) via different molecular targets. This is in line with previous clinical positron emission tomography (PET) studies (Bossong et al., 2009, Brody et al., 2009). In an earlier review, the authors reported that the DA increase parallels the euphoria that subjects experience (Volkow et al., 2009). Also, of importance is the pharmacokinetic profile of the substance, as a quick entry into the brain will also produce a stronger reinforcing effect as opposed to slow entry (reviewed by (Volkow et al., 2011)). However, this process is seen within normal and addicted subjects when presented with drugs of abuse, so it is important to highlight where and how DA projections are altered with respect to each drug to show the relationship with addiction (Volkow et al., 2011). Firstly, not only does a substance prompt an increase in DA within the brain, but a related stimulus (such as environmental cues) to this substance can also induce DA increases as it will provoke motivation in order to obtain the substance (Owesson-White et al., 2009). This is seen in a conditioning phase of studies; in order for conditioning to be successful only a stimulus (paired with a substance) should induce an increase of DA within the striatum/NAcc due to the anticipation created by the stimulus (Owesson-White et al., 2009, Schultz, 2010).

#### 1.4.1.1 DAT

The DAT is situated on the presynaptic plasma membrane of nerve terminals and is a key regulator of DA transmission in the CNS (Chen and Reith, 2000). This plasma membrane protein is responsible for DA clearance from the extracellular space by removing DA via reuptake and repackaging it into synaptic vesicles (Lohr et al., 2017, Sotnikova et al., 2006). This will then subsequently terminate any action of DA at its receptors (Wang et al., 2015), which in turn will result in associated changes in goal-directed motivation as well as in selective reinforcement (reviewed in (Arias-Carrión et al., 2010)).

Importantly, DA in the extracellular space can also be metabolized by MAO (Pereyra-Muñoz et al., 2006) or catechol-o-methyltransferase (Dichter et al., 2012), while former metabolism is associated with oxidative processes (Hermida-Ameijeiras et al., 2004). Indeed, the accumulation of DA in these vesicles (caused by DAT) can lead to oxidative stress and ultimately cause neurotoxicity (Masoud et al., 2015).

#### SCZ

DAT alterations found in SCZ subjects include;

- A post-mortem study found that SCZ patients have a decrease in DAT densities in the striatum (Dean and Hussain, 2001). According to these authors, this outcome may be based on two possibilities; one being a loss of the DAT molecule per se or secondly due to a reduction in innervating DA-ergic neurons.
- An imaging study found no differences in DAT densities when comparing treatment naïve SCZ patients with healthy subjects (Laakso et al., 2000). However, the authors did find that SCZ patients lack asymmetry in the caudate with regards to DAT binding (Laakso et al., 2000). This may be an indication of both functional and structural impairment of the lateralization of DA-ergic neurons that projects towards the caudate. (Laakso et al., 2000). This is in line with altered brain lateralization in SCZ (Laakso et al., 2000), and with more recent imaging studies (Chen et al., 2011, Schmitt et al., 2006). Lavalaye and colleagues also found no alterations in DAT densities when comparing healthy controls to treatment naïve patients. However, their study also included SCZ patients receiving antipsychotic treatment, and where no changes were evident (Lavalaye et al., 2001). This led them to the conclusion that DAT is not altered by antipsychotic treatment and that the functional changes (with regards to the DA system) in SCZ are not attributed to altered DAT densities (Lavalaye et al., 2001).
- Interestingly, an imaging study found that the severity of hallucinations experienced by treatment naïve patients are inversely correlated with DAT availability (Schmitt et al., 2006).

### *Drug abuse and addiction*

Drugs of abuse (e.g. cocaine) have the ability to bind to the DAT, and when bound, DAT is unable to take up excess DA from the synapse (dela Peña et al., 2015). Thus, these drugs disable a crucial process in regulating DA homeostasis that is essential in regulating DA-dependent cognitive brain function (Zhu et al., 2018).

DAT alterations found in addiction and drug abuse;

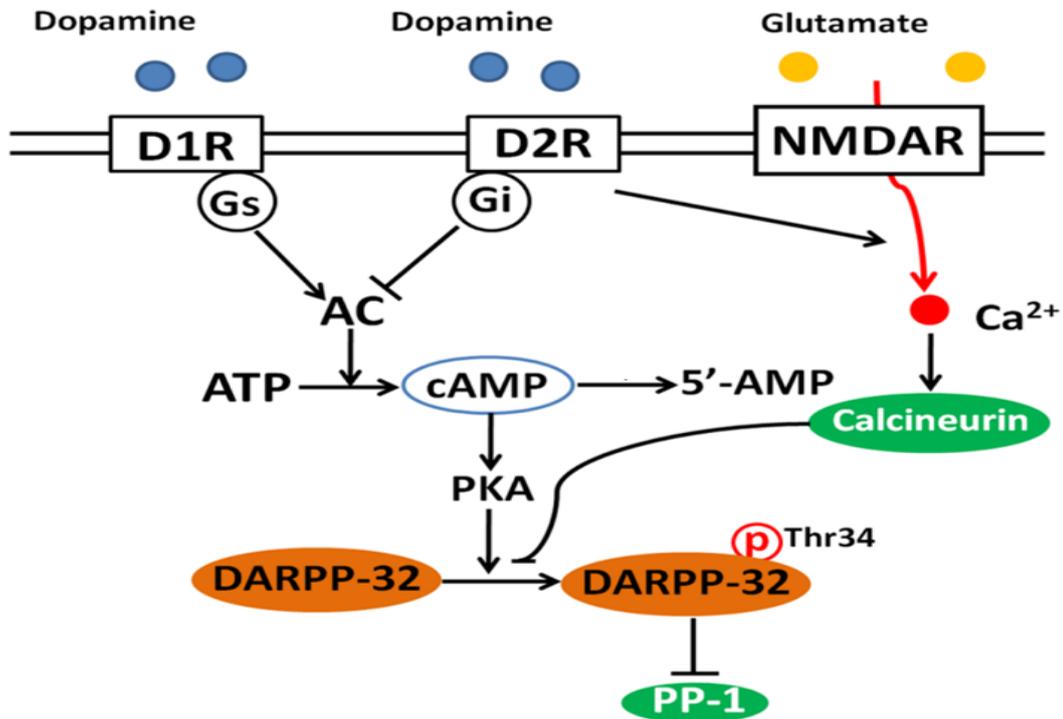
- Clinical findings have found that opioid-dependants have a lower striatal DAT (Liang et al., 2017) whereas cocaine-dependants show a higher DAT level when compared to control groups (Crits-Christoph et al., 2008)
- An imaging study found that drug users (in this case cannabis and tobacco) present with lower DAT densities within the dorsal striatum compared to healthy subjects (Leroy et al., 2012).

#### 1.4.1.2 DA-and- cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein (with a molecular weight of 32kD) (DARPP-32)

As mentioned above, DA pathways are essential for numerous functions and involved in SCZ, addiction and drug abuse. Alterations in these pathways can be regulated via interactions with D<sub>1</sub>-like and D<sub>2</sub>-like receptors (Missale et al., 2010), especially via phosphorylation. One target for the phosphorylation activity of DA receptors is DARPP-32 or otherwise known as phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1) (Wang et al., 2017). This phosphoprotein is expressed in MSNs (Svenningsson et al., 2004) in the NAcc, caudate, putamen, cerebral and cerebellar cortex, all of which receive DA projections (Brene et al., 1995). More specifically, DARPP-32 is also expressed within subclasses of DA neurons containing D<sub>1</sub>, and is thus an excellent marker for striatal DA projection neurons (Petryszyn et al., 2017). However, although DARPP-32 is regulated by DA, other neurotransmitters (e.g. GLU and 5-HT) can also activate DARPP-32 (Wang et al., 2017, Svenningsson et al., 2002).

The modulation of DARPP-32 (see figure 3 for a visual representation) is as follows: DA released from the DA neurons binds to the D<sub>1</sub> receptor, where it activates Gs proteins resulting in an increase in cAMP (Dichter et al., 2012) and activation of protein kinase A (PKA) (Wang et al., 2017). PKA phosphorylates DARPP-32 at threonine 34 (Thr34), a process that regulates striatal function (Dichter et al., 2012, Wang et al., 2017). DARPP-32 is a potent protein phosphatase 1 (PP-1) inhibitor (in the phosphorylated state (Hemmings Jr et al., 1984)) which is essential to DA and GLU signalling as well as integrating these two pathways (Albert et al., 2002). By inhibiting PP-1, DARPP-32 controls the activity of ion channels, - pumps, transcription factors, and neurotransmitter receptors (Albert et al., 2002). Thus, it can control neurons containing DA receptors by controlling the neuron's physiological traits (Albert et al., 2002). Also, the inhibition

of PP-1 will result in the inhibition or reduced phosphorylation of the proteins that are implicated in synaptic plasticity and function (Svenningsson et al., 2004). On the other hand, activation of the D<sub>2</sub> will inhibit DARPP-32 phosphorylation as the activation of the receptor will lead to the inhibition of adenylate cyclase and cAMP (Wang et al., 2015). Furthermore, the activation of the N-methyl-D-aspartate glutamine (NMDA) receptor (NMDAR) will result in an increased calcium (Ca<sup>2+</sup>) level which will subsequently activate calcineurin. This process will in turn cause a dephosphorylation of DARPP-32 at Thr34.



**Figure 3:** The modulation of dopamine and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein (with a molecular weight of 32kD) (DARPP-32) - (Wang et al., 2017). Arrows represent activation and blocked lines an inhibition. Abbreviations; D1R – DA 1 receptor, D2R – DA 2 receptor, NMDAR - N-methyl-D-aspartate glutamine receptor, AC – adenylate cyclase, ATP – Adenosine Triphosphate, cAMP – cyclic adenosine monophosphate, 5'-AMP – 5' adenosine monophosphate, PKA – protein kinase A, PP-1 – protein phosphatase 1, Thr34 – Threonine 34.

### SCZ

The modulation of DARPP-32 in SCZ patients can be attributed to the DA and GLU hypotheses of this disorder. Firstly, a dysfunctional mesocorticolimbic DA pathway characterized by regional increased DA as well as DA receptor expression levels, contribute towards this modulation (Wang et al., 2017). An animal study found increased DARPP-32 activity to follow as a result of chronic hyperdopaminergia (Wang et al., 2017). A clinical study, on the other hand, found that DARPP-

32 was decreased within the PFC of SCZ patients and could explain compromised cognitive functioning in these patients (Albert et al., 2002). This study is in line with another pre-clinical study that found that the DARPP-32 expression levels correlated positively with cognitive performance (Kolata et al., 2010). These findings may be due to SCZ patients experiencing hypodopaminergic transmission within the PFC (Howes and Kapur, 2009), therefore suggesting that lowered DA levels can cause lowered DARPP-32 expression in the PFC. Secondly, pre-clinical studies have confirmed the effects of GLU on the modulation of DARPP-32. Indeed, exposure to PCP in mice deficient in DARPP-32 in GABA MSN enhances DARPP-32 phosphorylation in the striatum (Pozzi et al., 2010). These KO animals did not present with any motor effects induced by PCP, although memory deficits remained (Bonito-Oliva et al., 2016). Interestingly, the antagonistic modulation of DARPP-32 via the NMDAR and D<sub>2</sub> (Albert et al., 2002) is critically involved in synaptic plasticity and GLU transmission, in which DARPP-32 activation is needed for long term potentiation, a process involved in memory and learning (Calabresi et al., 2000).

#### *Drug abuse and addiction*

DARPP-32 plays an important part in mediating and modulating the short- and maybe the long-term actions of abusive drugs (Svenningsson et al., 2005). A pre-clinical study found that drugs of abuse such as cocaine and amphetamine increased the levels of DARPP-32 phosphorylation within the nigrostriatal region (Svenningsson et al., 2005). Supporting this statement, DARPP-32 KO mice show a decreased response towards cocaine place preference (Zachariou et al., 2002). Given the earlier mentioned effects of DA metabolism on cellular redox state, increased DA induced by drugs of abuse (reviewed by (Yger and Girault, 2011)) can lead to increased levels of oxidative stress (Pereyra-Muñoz et al., 2006). Furthermore, this process may in turn lead to an increase in DARPP-32 levels within the striatum as well as the substantia nigra (Pereyra-Muñoz et al., 2006).

#### 1.4.2 5-HT

##### *SCZ*

The role of 5-HT in SCZ has been proposed since the 50's where evidence connected serotonergic dysfunction to several symptoms experienced by SCZ patients (Selvaraj et al., 2014, Meltzer and Sumiyoshi, 2008). This neuromodulator is also critically involved in other psychiatric conditions such as anxiety, depression and obsessive compulsive disorder (Dayan and Huys, 2008).

Early studies focussed on the link between hallucinogenic drugs (LSD, psilocybin and dimethyltryptamine) and their activation of cortical 5-HT<sub>2A</sub> receptors (Eggers, 2013, Aghajanian

and Marek, 2000). This interaction will ultimately result in distorted perceptions, disordered cognition and paranoia (similar to SCZ symptoms) (Geyer and Vollenweider, 2008). In support of this, other 5-HT<sub>2</sub> agonists (e.g. m-chlorophenylpiperazine) aggravate psychotic symptoms in SCZ patients (Abi-Saab et al., 2002).

It is however noteworthy to report that altered 5-HT function will also implicate altered GLU & GABA signalling thereby connecting several hypotheses of SCZ (reviewed by (Quednow et al., 2010, Meltzer and Sumiyoshi, 2008)).

### *Drug abuse and addiction*

The 5-HT system is essential for regulating reward-related behaviours such as drinking, eating and sexual behaviours. This is in line with numerous animal data (see (Pfaus, 2009, Wirtshafter, 2001) for reviews) as well as clinical studies (reviewed by (Cools et al., 2008)). 5-HT-ergic cell bodies in the raphe nuclei (RN) (Hayes and Greenshaw, 2011) innervate most of the 5-HT projections in the forebrain (Azmitia and Segal, 1978) as well as in the brain reward areas (i.e. NAcc, VTA, SN, HPC, PFC and amygdala) (Hensler, 2006, Lechin et al., 2006, Ikemoto, 2010). Pre-clinical studies have found that stimulation of the 5-HT rich RN can bolster intracranial self-stimulation (Broadbent and Greenshaw, 1985, Van Der Kooy et al., 1978) while substances that increase 5-HT in the brain may increase a reward within the conditioned place preference (CPP) paradigm (Subhan et al., 2000). For an extensive review on how psychostimulants affect 5-HT levels within respective brain areas, refer to (Müller et al., 2007). 5-HT also has the ability to regulate various neurotransmitters implicated in reward, most specifically DA (for a review, see (Fink and Göthert, 2007)).

The complexity of the 5-HT system lies in that 5-HT acts on numerous receptor subtypes, while the role of 5-HT in reward is heavily dependent on the receptor subtype (Hayes and Greenshaw, 2011). These distinct roles are possibly due to their effects on ionic conductance, second messengers and their specific distribution throughout the CNS (Hayes and Greenshaw, 2011). During the past years, several researchers have highlighted the specific role of 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptors in addiction and reward. Therefore, only these two receptors will be discussed, but for an extensive review on the other subtypes please refer to (Hayes and Greenshaw, 2011, Kranz et al., 2010).

The 5-HT<sub>1A</sub> receptor is found in various brain areas (i.e. dorsal RN, HPC, amygdala) both pre- (autoreceptors) and postsynaptically (Van Wijngaarden et al., 1990) (Müller et al., 2007). The stimulation of the 5-HT<sub>1A</sub> autoreceptors inhibits 5-HT cell firing (in the RN) and result in reduced 5-HT production and release at end synapses (reviewed by (Müller et al., 2007)) which may influence an individual's susceptibility towards mood disorders (Albert et al., 2011). Postsynaptic 5-HT<sub>1A</sub> receptors located in the mesocorticolimbic areas of the brain are of particular importance

for the effects induced by psychostimulants (Müller et al., 2007). Here for example 5-HT<sub>1A</sub> agonists can lead to the release of DA in the NAcc, striatum and frontal cortex (Benloucif and Galloway, 1991, Ago et al., 2003) with resulting behavioural effects. Furthermore, 5-HT<sub>1A</sub> hyperactivation by psychostimulants and their long-term exposure may result in altered receptor densities and function that contribute towards addictive-like behavioural effects (Müller et al., 2007).

5-HT<sub>2A</sub> receptors are more implicated in the psychogenic effects of psychedelic- and hallucinogenic substances like LSD. In fact, studies in rodents show that activation of 5-HT<sub>2A</sub> leads to a distinct behavioural response (head-twitch or wet dog shake) (Halberstadt and Geyer, 2013), while such agents evoke hallucinogenic effects in humans (González-Maeso et al., 2007). Furthermore, 5-HT<sub>2A</sub> antagonists can block these behavioural effects in both animals and humans (Halberstadt and Geyer, 2013).

Gatch et al., (2013) indicated that EFV has possible LSD-like properties and we will, therefore, focus on the neurochemistry of LSD.

LSD acts as an agonist on the G-protein-coupled 5-HT<sub>2A</sub> (Kyzar et al., 2017) which is present in the endocrine -, immune system, the brain and several other tissues (Kyzar et al., 2017). LSD activation of 5-HT<sub>2A</sub> receptors in the RN results in GLU release via the thalamic GLU-ergic neurons resulting in hyper GLU transmission within the PFC (Ham et al., 2017). Hyper GLU transmission in the PFC (Ham et al., 2017) leads to DA release in the NAcc (Murase et al., 1993), thereby inducing the euphoric effects of LSD. The recreational use of LSD may precipitate the onset of psychosis (Carhart-Harris et al., 2016) which is similar to that seen in psychotic SCZ patients (Nichols, 2004).

## 1.5 Neurodevelopmental hypothesis

### SCZ

The neurodevelopmental hypothesis of SCZ is especially intriguing as it suggests that the disorder manifests due to aberrant brain development during the pre-natal or post-natal period (Rehn and Rees, 2005). This hypothesis ultimately suggest that any adverse event or insult during the in utero period may alter normal brain development in the foetus and create a vulnerability and predisposition for SCZ within the individual (Rehn and Rees, 2005). Investigative studies have also proposed significant evidence that post-natal insults or events (e.g. substance abuse, social- and economic status, living conditions) may also contribute towards this manifestation (Rehn and Rees, 2005).

Pre-natal insults include; low birth weight, gestational diabetes (Cannon et al., 2002), reduced maternal exposure to sunlight (vitamin D deficiency) (McGrath et al., 2002), and nutritional

deprivation (Brown et al., 1996). But more importantly for this study, maternal infections (viral (Brown et al., 2004, Mednick et al., 1988) or bacterial (Sørensen et al., 2008)) especially during the first two trimesters of pregnancy (Rehn and Rees, 2005) are a significant predisposing factor. A recent review evaluated 36 studies (clinical, pre-clinical and neuroimaging), showing a distinct correlation between in utero infections and SCZ (Guma et al., 2019).

Importantly, the link between maternal infection during pregnancy and the development of SCZ does not depend on the exposed pathogen, but rather the induced maternal immune activation (MIA) (discussed in section 1.11.1) and the subsequent generation of an inflammatory response (discussed in section 1.6) within the foetus (Guma et al., 2019).

### *Drug abuse and addiction*

Drug abuse and addiction is not characterised as a neurodevelopmental disorder per se, but there is good evidence linking neurodevelopmental factors to these conditions.

- Pre-clinical studies determined that pre-natal stress enhances a response to cocaine (Kippin et al., 2008, Bolton et al., 2018), alcohol (Campbell et al., 2009), amphetamine (Hausknecht et al., 2013), benzodiazepines (Lakehayli et al., 2015), opioids (Yang et al., 2006) and nicotine (Said et al., 2015).
- Pre-clinical studies have determined that MIA may also contribute towards addictive behaviours later in life (Borçoi et al., 2015, Straley et al., 2017).
- A case-control study found that pre-natal exposure to famine may be associated with an addiction disorder later in life (Franzek et al., 2008).
- Clinical studies have found that factors such as childhood neglect and household dysfunction (Dube et al., 2003) as well as child abuse (Marcenko et al., 2000) and childhood sexual abuse (Wilsnack et al., 1997) may also contribute towards the development of addiction.

## **1.6 Inflammation**

Inflammation induced by a pathogen and the resulting activation of the immune system (Mongan et al., 2019) is promoted by proinflammatory factors i.e., proinflammatory cytokines (especially tumour necrosis factor (TNF)- $\alpha$  & interleukin (IL)-1 $\beta$ , IL-6, IL-8), prostaglandins, chemokines and leukotrienes (for a full review on each main immunologic effect, see (Meyer, 2013)), all crucial effectors of an immune response (Borish and Steinke, 2003). Cytokines are the immune system's signalling molecules and have the ability to cross the BBB (Miller et al., 2013). Once the signal has reached the brain, the rich cytokine network orchestrated by microglia, astrocytes and

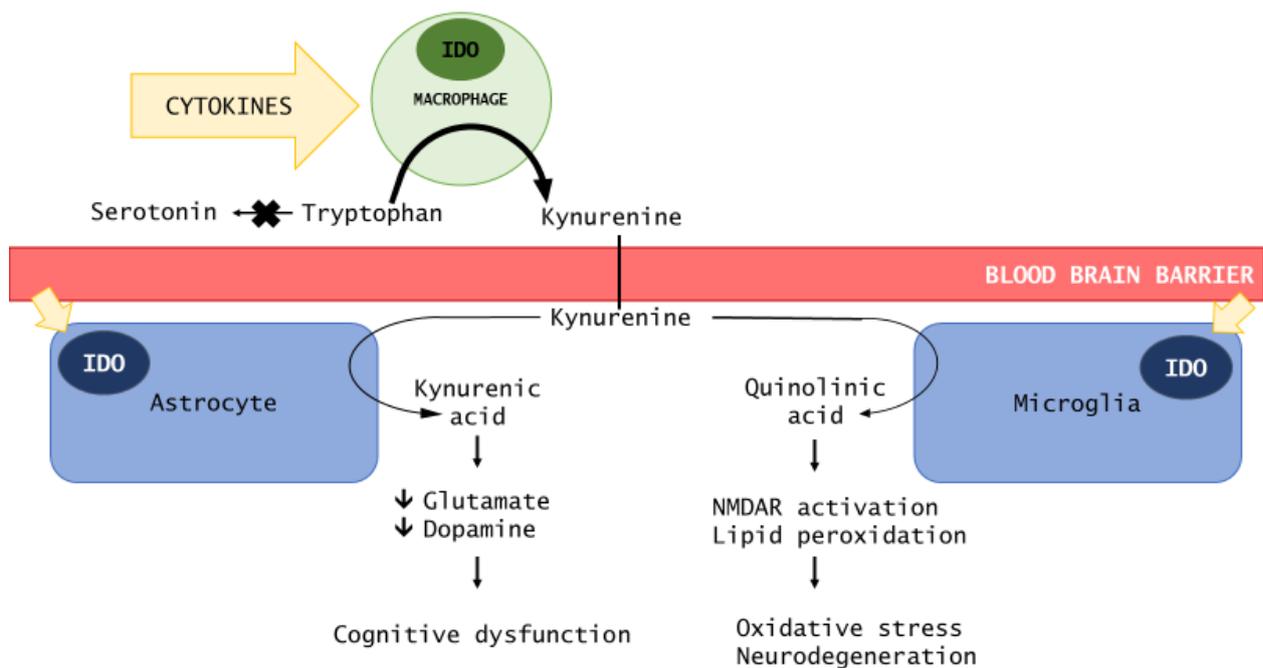
neurons will produce more cytokines as well as promote the expression of cytokine receptors (Miller et al., 2009). The microglia in particular are also activated by stress (Frank et al., 2007).

## SCZ

Various mechanisms are proposed whereby cytokines alter neurotransmitters, receptors, glial elements and other factors, all of which can be connected to the development and behavioural profile of SCZ.

- A recent meta-analysis found increased IL-6 & 8 in the cerebrospinal fluid (CSF) of SCZ patients compared to the healthy patients (Orlovska-Waast et al., 2019).
- The kynurenine pathway plays a key role in the inflammatory response, and is connected to monoamine metabolism (reviewed by (Haroon et al., 2012, Schwarcz and Pellicciari, 2002) see figure 4 for a visual representation).

Indolamine-2,3-dioxygenase (IDO), responsible for synthesizing kynurenine from tryptophan, is expressed in various immune cell types such as microglia, astrocytes, dendritic cells, neurons and macrophages. Inflammatory cytokines activate this enzyme, which ultimately affects the synthesis of 5-HT from tryptophan, its primary precursor. Furthermore, kynurenine is catabolized to quinolinic- (QA), an NMDA agonist, and kynurenic acid, an NMDA antagonist. The latter can reduce GLU release, by virtue of its NMDAR antagonist properties, thereby also affecting DA release. QA also acts as a NMDAR agonist, resulting in an increased GLU release, thereby provoking neurotoxicity and oxidative stress.



**Figure 4:** Indolamine-2,3-dioxygenase (IDO) and kynurenine pathway altered by cytokine exposure – Adapted from (Haroon et al., 2012). Cytokines have the ability to activate IDO on peripheral- or brain immune cells. This will lead to the production of kynurenine which will be converted to either kynurenic- or quinolinic acid. Both conversions will have an effect on monoamines, as well as introduce other factors such as oxidative stress. Abbreviation: NMDAR - N-methyl-d-aspartate receptor.

Other findings in SCZ patients connected to the above-mentioned process include:

Microglial densities are increased in the post mortem SCZ brain compared to healthy subjects (Van Kesteren et al., 2017).

PET studies established microglial activation within SCZ patients as well as high-risk (for SCZ) patients (Bloomfield et al., 2015).

A clinical study reported disordered tryptophan metabolism in SCZ patients that can be linked to the positive symptoms they experience (Kim et al., 2009).

- Toll-like receptors (TLR)

TLR's are pattern recognition receptors that are a part of the innate immune system and are responsible for identifying microorganisms (Takeuchi and Akira, 2010). Upon activation (via pathogenic molecules), TLR's stimulate the production of cytokines and other proinflammatory molecules (Takeuchi and Akira, 2010). Importantly, over-activity of the TLR system is associated with psychotic episodes (reviewed by (Mongan et al., 2019)). Other alterations of TLR's found in SCZ include:

TLR-4 is overexpressed in the brains of post mortem SCZ patients (MacDowell et al., 2017).

TLR agonist induced release of cytokines (IL-1 $\beta$ , IL-6, IL-8 & TNF- $\alpha$ ) are higher in SCZ patients compared to that in healthy subjects (McKernan et al., 2011).

### *Drug abuse and addiction*

Inflammatory alterations as induced by drugs of abuse;

- Alcohol induces nuclear factor kappa light chain enhancer of activated B cells (NF $\kappa$ B) activity (Davis and Syapin, 2004, Zou and Crews, 2010). NF $\kappa$ B is an important mediator in the innate immune response that is activated upon TLR<sub>4</sub> activation (Rivest, 2003) (This is further explained in section 1.11.1).
- MA enhances the activation of NF $\kappa$ B and activator protein 1 (AP-1) (Lee et al., 2001). Upon activation of these transcription factors, it induces the expression of several inflammatory mediators which may be crucial for the development of addiction (Rodrigues et al., 2014).

- A pre-clinical study found that chronic alcohol binge will increase the response to lipopolysaccharide (LPS) as well as cytokine expression (Qin et al., 2008).
- A review study found the following immune alterations as induced by cocaine (Ersche and Döffinger, 2017);
  - Increased IL-6, IL-1 $\beta$ , TNF- $\alpha$  as well as decreased IL-10 serum levels when compared to control groups.
- Psychostimulants induce cytokine expression as well as microglial activation which may facilitate the development of addiction (Clark et al., 2013, Sadasivan et al., 2012).
- MA increases TNF- $\alpha$  (Nakajima et al., 2004, Yamada and Nabeshima, 2004) in humans and animals.
- Amphetamines have the ability to decrease lymphocytes but increase neutrophils in animals (Llorente-Garcia et al., 2009).

### **1.7 The role of the cerebellum in SCZ and addiction**

The cerebellum is located at the base of the brain (Andersen et al., 1992) and functionally divided into three parts, i.e. cerebral hemisphere, vermis and flocculonodular lobe (Triulzi et al., 2005), all responsible for certain motor behaviours (Hoppenbrouwers et al., 2008). The flocculonodular lobe is connected to the spinal cord and brain stem and supports functions such as posture and balance, whereas the vermis is responsible for movement and muscle tone (Hoppenbrouwers et al., 2008). Interestingly, the cerebral hemispheres project towards the cerebral cortex and are connected to the dentate nucleus (Hoppenbrouwers et al., 2008). The latter connection links the influence of the cerebellum to cognitive function (Hoppenbrouwers et al., 2008). Also, a clinical study determined that an emotional stimulus has the ability to activate the cerebellum (Habel et al., 2005), while various neuropsychiatric disorders present with functional and/or structural alterations of the cerebellum (Villanueva, 2012).

#### **SCZ**

The role of the cerebellum in SCZ is well-recognized (Andreasen and Pierson, 2008, Yeganeh-Doost et al., 2011) and it is hypothesized that cerebellar dysfunction may result in cognitive deficits (Villanueva, 2012). This correlates with clinical- (Forsyth et al., 2010) and review (Andreasen et al., 1998) studies proposing a possible disruption in the cortical-subcortical-cerebellar circuitry in the illness, which is supported primarily by protein- and gene expression studies using synaptic markers (i.e. synaptophysin, complex II and semaphoring 3A (Eastwood et al., 2003)). However, several clinical- and imaging studies have also presented other very persuasive evidence;

- SCZ patients present with a reduced vermis volume (Greenstein et al., 2011, Ichimiya et al., 2001). An early clinical study found that this atrophy correlated significantly with the psychopathology of SCZ (Sandyk et al., 1991).
- SCZ patients present with a reduced gyrification index (Schmitt et al., 2011) and loss of Purkinje cells within the cerebellar cortex along with thinning of molecular and granular layers in the vermis (Weinberger et al., 1980).
- Increased D-amino acid oxidase expression and activity in SCZ patients (Verrall et al., 2007), an enzyme that regulates the activation of NMDAR via the modulation of D-serine availability (Burnet et al., 2008). This is in line with the NMDAR deficit hypothesis (Schmitt et al., 2010).
- Reduced N-acetyl aspartate (NAA) levels in the cerebellar vermis, cortex and thalamus of SCZ patients (Deicken et al., 2001, Ende et al., 2005). NAA is a biomarker for neuron viability and –density (Deicken et al., 2001).
- The reader is also referred to the following review article (Hoppenbrouwers et al., 2008) that elaborates further on the cerebellum and its link to the pathophysiology of SCZ.

### *Drug abuse and addiction*

Recent studies have highlighted the cerebellum for its involvement in addiction (see (Miquel et al., 2016, Moulton et al., 2014) for a review), where a definite relationship between the cerebellum and cortical- as well as subcortical areas are implicated in addiction, but more specifically in craving (Moreno-Rius and Miquel, 2017). In fact, addictive substances such as alcohol (Freund and Palmer, 1997, Netzeband et al., 1999) and psychostimulants (Vazquez-Sanroman et al., 2015)) directly alter cerebellum plasticity and the functioning thereof (Moreno-Rius and Miquel, 2017). A clinical study found decreased grey matter in the cerebellum following cocaine use (Moreno-López et al., 2015) while another clinical study described cerebellar activation following presentation of a drug-associated cue to users (Filbey et al., 2010). Also noteworthy is that degeneration of the cerebellum is linked to cognitive and emotional deficits following long-term exposure to substances such as alcohol (Fitzpatrick et al., 2008). A pre-clinical study observed that cocaine increased mononuclear phagocytes within the cerebellum (López-Pedrajas et al., 2015), which has been suggested to be due to drug-related neurotoxicity caused by excessive GLU levels. Cerebellar alterations also occur in cannabis users where these changes are linked to the ability of cannabis to induce psychotic symptoms as well as the appearance of SCZ (Villanueva, 2012). These findings are also in line with a clinical study of Dervaux and colleagues (Dervaux et al., 2013).

### **1.8 Neuroplasticity**

Neuroplasticity defines the ability of the brain to adapt (positively or negatively) its connections, structure or function following exposure to novel experiences, diseases or changes in the

environment (Wynn et al., 2019, Frost et al., 2004). One form of neuroplasticity is long-term potentiation (LTP), which refers to a long term (minutes/hours) augmentation of excitatory synaptic transmission (Cooke and Bliss, 2006, Bliss and Collingridge, 1993). This is characterized as the major cellular mechanism needed for learning and memory (Cooke and Bliss, 2006, Bliss and Collingridge, 1993). Importantly, LTP is very dependent on GLU transmission at the NMDAR (Malenka and Bear, 2004, Cooke and Bliss, 2006). GLU and NMDAR dysfunction is present in both SCZ (Moghaddam and Javitt, 2012) and addiction (D'Souza, 2015).

## SZ

Neuroplasticity that occurs in SCZ may subsequently result in changes in certain neural networks along with molecular changes responsible for modulating neurotransmitter receptor function (Frost et al., 2004, Lewis and González-Burgos, 2008). Also, LTP neuroplasticity could explain neural processes connected to the positive- (Hoffman and Cavus, 2002, Port and Seybold, 1995) as well as the cognitive symptoms (Forsyth and Lewis, 2017, Cooke and Bear, 2012) experienced by SCZ patients. Interestingly, a clinical study have found that neuroplasticity-based cognitive training can improve cognitive control as well as memory and learning in patients with SCZ (Fisher et al., 2009).

### *Drug abuse and addiction*

The transition from drug abuse to addiction may be attributed to neuroplastic changes within certain brain circuitries that will ultimately enhance learned drug-associated behaviours (Kalivas and O'Brien, 2008). Importantly, plasticity changes associated with addiction alone as well as changes induced by certain types or classes of drugs of abuse, are observed (Kalivas and O'Brien, 2008). Drugs of abuse have the ability to interact and change brain circuitries that facilitate learned and adaptive behaviours in response to environmental stimuli (Everitt and Robbins, 2005, Kelley, 2004). By changing these brain circuitries, drugs of abuse will damage the development of behavioural strategies towards the stimuli, therefore altering drug-seeking as well as drug-taking strategies (Kalivas and Volkow, 2005). For a comprehensive review on neuroplasticity in all stages of addiction, please see (Kalivas and O'Brien, 2008).

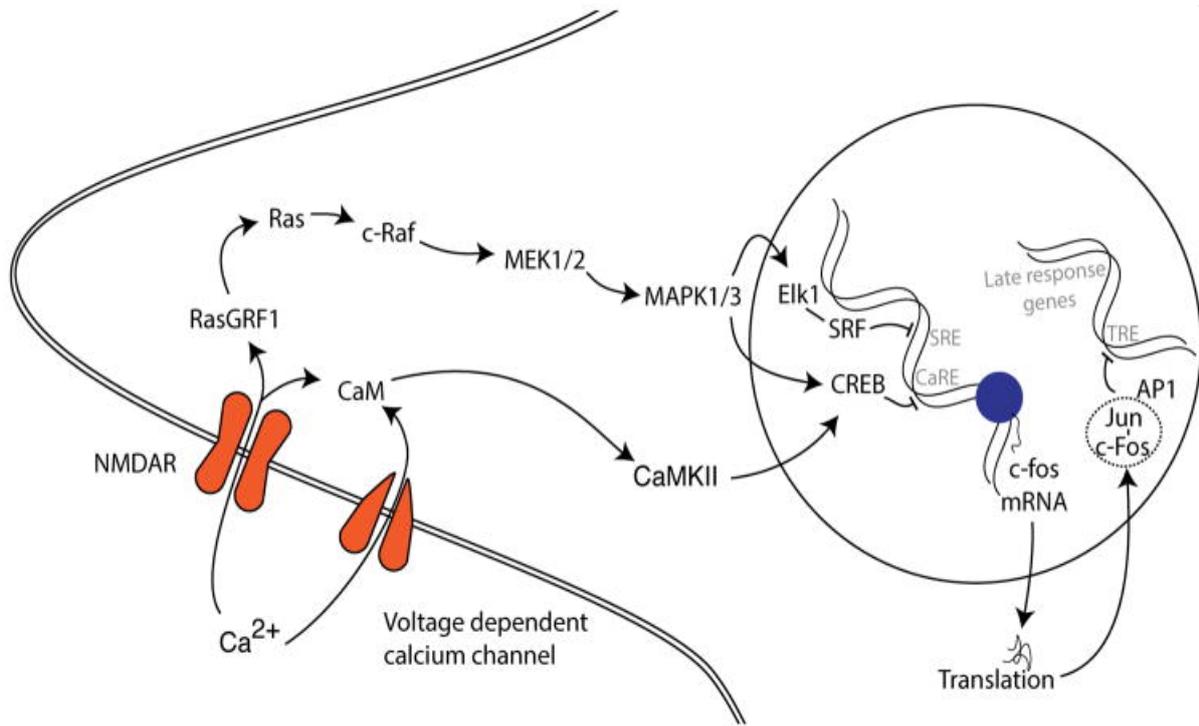
#### 1.8.1 c-Fos

c-Fos is an immediate early gene (IEG) used as a functional mapping tool to identify circuitries and cells that are activated in response to various physical, emotional and pharmacological stimuli (reviewed by (Kovács, 1998)). Physiological stimuli include ultraviolet radiation, cytokines, serum, tumour promoters and growth factors (Bahrami and Drabløs, 2016). IEGs are regulated through neural activity and serve as a marker for behaviour and cognition (Gallo et al., 2018). Thus increased expression of IEGs are used to indirectly measure neural activity within experimental

subjects (Gallo et al., 2018). In this regard, c-Fos expression is connected to memory and learning (especially those associated with neuropsychiatric disorders) as any change in expression is selectively and swiftly upregulated within the brain regions connected to memory and learning formation (Minatohara et al., 2016).

In the 80's Leszczek Kaczmarek proposed a hypothesis which connected c-Fos expression to synaptic plasticity (Jaworski et al., 2018). Kaczmarek proposed that any alterations within neuronal gene expressions that are mediated by transcription factors should be responsible for any changes that cause memory, learning and plasticity. Transcription factors regulate genetic programs and subsequently result in functional and structural remodelling of neurons by altering synaptic strength, dendritic spine shapes and number of neuronal connections (Jaworski et al., 2018). This will in turn lead to balanced neuroplasticity and connectivity, two factors which are essential for memory and learning (Jaworski et al., 2018). Importantly, c-Fos can be induced when exposed to conditions involving long-term neuronal plasticity (including memory and learning) (Jaworski et al., 2018), e.g. behavioural training regimes result in increased expression of c-fos within certain brain regions (see the following articles for examples (Kaczmarek, 1992, Tischmeyer et al., 1990)). A pre-clinical study done in 2017 implicated c-Fos in experience-dependant learning and plasticity (de Hoz et al., 2017).

The signal transduction pathway to c-Fos is directly linked to NMDAR-mediated  $Ca^{2+}$  influx and/or activation of voltage dependant calcium channels (Hudson, 2018) leading to mobilisation of the calcium/calmodulin dependant protein kinase II and cAMP-response element binding protein cascade (Hudson, 2018). This process will ultimately result in the expression of c-fos messenger ribonucleic acid which can be transcribed to c-Fos proteins, with the latter binding to Jun to form the transcription factor AP-1 (Hudson, 2018). AP-1 can transcribe late response genes via 12-O-Tetradecanoylphorbol-13-acetate response element (Hudson, 2018). AP-1 is a positive modulator of the extracellular operating endopeptidase, matrix metalloproteinase 9 (MMP-9), implicated in neuronal plasticity (Meighan et al., 2006, Nagy et al., 2006, Michaluk et al., 2007, Okulski et al., 2007, Jasińska et al., 2016, Szepesi et al., 2014, Kondratiuk et al., 2017, Michaluk et al., 2011). Importantly, MMP-9 is implicated in psychiatric disorders such as SCZ (Lepeta et al., 2017) and alcoholism (Samochowiec et al., 2010).



**Figure 5:** The signalling transduction pathway to c-Fos – (Hudson, 2018). Abbreviations: NMDAR – N-methyl-D-aspartate glutamine receptor, RasGRF1 – Ras-guanine nucleotide releasing factor, MEK 1/2 – mitogen activated protein kinase 1 and 2, MAPK 1/3 – mitogen-activated protein kinase 1 and 3, CREB, cyclic AMP response element-binding protein, CaRE – cAMP response element, SRF – serum response factor, SRE – serum response element, CaM – calmodulin, CaMKII – calcium/calmodulin dependant protein kinase II, AP1 – activating protein 1, TRE – 12-O-Tetradecanoylphorbol-13-acetate response element.

## SCZ

c-Fos expression is altered in cortical regions (associated with cognitive processing) in a NMDAR antagonist (e.g. MK-801) induced animal model of SCZ (Vishnoi et al., 2015). In addition to c-fos changes, the researchers also found deficits in conditioned taste aversion, novel object recognition and an increase in locomotor activity (Vishnoi et al., 2015). Data regarding c-Fos alterations in the cerebellum, with regards to psychosis and SCZ, are extremely limited, but a summary of the available studies show:

1. An increase in c-Fos after PCP exposure (Shimizu et al., 1997).
2. An increase in c-Fos after PCP (50 mg/kg) exposure (Näkki et al., 1996). Thus, the psychotogenic agent, PCP, produces neural activation within the cerebellum (Näkki et al., 1996).

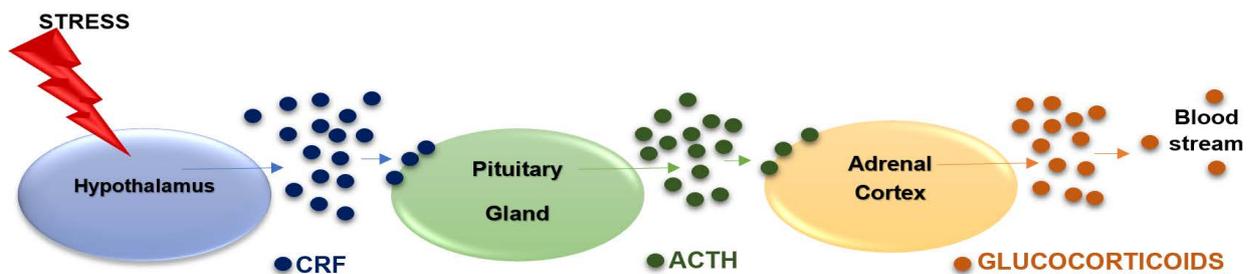
## Drug abuse and addiction

Studies exploring the role of the cerebellum in addiction, and the functional significance of cerebellar activation, are limited, but can be summarised as follows:

1. Cocaine-induced preference conditioning in rats is associated with increased c-Fos expression in the cerebellum (Carbo-Gas et al., 2014).
2. Alcohol withdrawal-induced c-Fos expression is reduced in the cerebellum and HPC in rats (Putzke et al., 1996).
3. Increased levels of reactive oxygen species (ROS) in the cerebellum correlate with increased levels of c-Fos expression, and can be reversed by administering an anti-oxidant such as N-acetyl cysteine (NAC) (Cheng et al., 1999).

### 1.9 Hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis is a vital system responsible for mediating the stress responses in mammals (Corcoran et al., 2003). The HPA axis consists of three vital structures, namely the hypothalamus, the pituitary gland (anterior lobe) and the adrenal gland (Turnbull and Rivier, 1997), and can be activated via external stressors (Binder and Nemeroff, 2010). The hypothalamus has neurosecretory neurons that produce and release corticotropin-releasing factor (CRF), which then binds to the CRF<sub>1</sub> receptor on pituitary corticotropes to stimulate the release of adrenocorticotrophic hormone (ACTH) into the circulation (see figure 6) (Liyanarachchi et al., 2017, Binder and Nemeroff, 2010). The ACTH in the circulation stimulates glucocorticoid (cortisol in humans, corticosterone (CORT) in rodents) production in the adrenal glands, which is released into the bloodstream (see figure 6) (Koob, 2008). Thereafter, the released glucocorticoid will exert various metabolic, immune and neuromodulator effects in the body, including amongst others affecting neurotransmitter systems (5-HT, GABA<sub>A</sub>, GLU and DA) (Joca et al., 2007, Kamin and Kertes, 2017).



**Figure 6:** Regulation of the Hypothalamic-Pituitary- Adrenal (HPA) axis – Adapted from Liyanarachchi et al. (2017) and Binder & Nemeroff (2010). Abbreviations: CRF – corticotropin-releasing factor, ACTH – adrenocorticotrophic hormone.

During inflammation, the production of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  is increased (Borish and Steinke, 2003, Tenk et al., 2013), which in turn is suppressed by cortisol/CORT; thus, cortisol is considered an immunosuppressant (Boumpas et al., 1993, Chrousos, 1995, Elenkov, 2008). However, in the case of chronic exposure to stressors (activation of the HPA axis), this effect can change to being immune modulatory (Elenkov, 2008).

Due to high levels of glucocorticoid released over a prolonged period, this leads to down-regulation of glucocorticoid receptors (Silverman and Sternberg, 2012) culminating in an altered capacity of the body to inhibit the inflammatory response and its response to cortisol (Chrousos, 1995). These modified changes in the immune-endocrine interactions have previously been linked to neuropsychiatric disorders such as SCZ (Walker et al., 2008) and depression (Lutgendorf et al., 2008), so much so that these illnesses are now regarded as a pro-inflammatory state (Brand et al., 2015, Schiepers et al., 2005). Furthermore, the possible molecular link between psychiatric disorders and HPA axis dysfunction appears to be oxidative stress (Şimsek et al., 2016).

## SCZ

Cortisol dysregulation, especially a hyperactive HPA axis (Walker et al., 2008), has been found in patients with SCZ and the levels thereof has been linked to cognitive deficits, psychosis as well as structural brain changes (Corcoran et al., 2003). This is in line with a pre-clinical study using a neurodevelopmental model of SCZ that found elevated levels of CORT in plasma along with decreased glucocorticoid receptor levels in the HPC (Basta-Kaim et al., 2011).

## *Drug abuse and addiction*

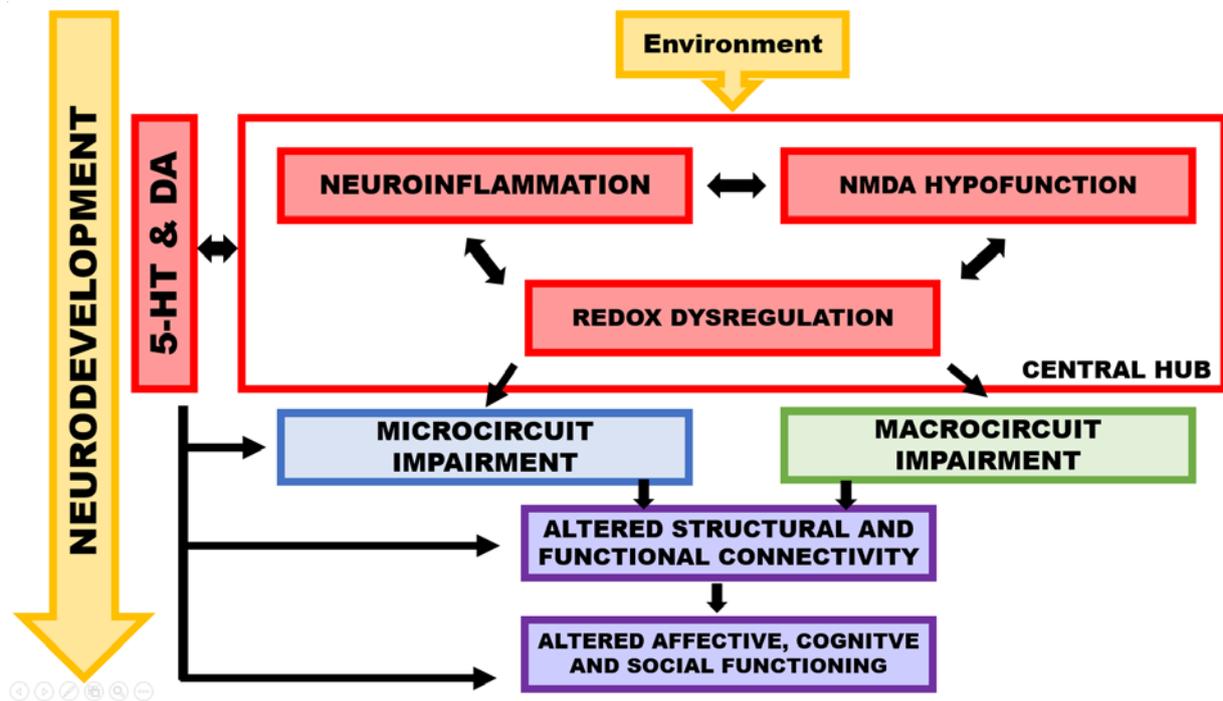
Previous studies have proposed that CRF can be linked to the relapse behaviour observed in drug addiction (see (Bruchas et al., 2010) for a review) as well as the vulnerability to developing drug addiction (Sinha, 2008). The administration of drugs of abuse can lead to the release of CRF within the central nucleus of the amygdala (Edwards and Koob, 2010), while the reward system can be stimulated when exposed to a stressor via activation of the HPA-axis (Bruchas et al., 2010).

## **1.10 Oxidative stress**

Oxidative stress is an imbalance that occurs between the production of ROS and reactive nitrogen species (RNS) and the removal thereof by an anti-oxidant system (Sayre et al., 2008, Wood et al., 2009). Failure to curb this process ultimately results in damage to macromolecules such as proteins and also the activation of redox-sensitive signals (Fung and Hardan, 2019). The brain is highly susceptible to oxidative stress due to numerous factors such as a high oxygen demand, the lack of sufficient antioxidant systems, excessive GLU uptake and the metabolism of endogenous amines such as DA, to name but a few (for a full review see (Cobley et al., 2018)). Glutathione (GSH), the predominant anti-oxidant in living organisms, exists in two forms, i.e. oxidised GSH (GSSH) and reduced GSH (Fung and Hardan, 2019) which can interact directly with ROS and RNS (Lushchak, 2012). Reduced GSH and GSSH both serve as excellent markers to measure the level of oxidative stress within a subject (Fung and Hardan, 2019).

## SCZ

Steullet and colleagues hypothesized that redox regulation, neuroinflammation and NMDA hypofunction form a central hub of SCZ pathophysiology (Steullet et al., 2010) (see figure 7 for an visual representation). These three components may mediate impairments of micro- (maturation of parvalbumin interneurons, excitatory/inhibitory balance) and macrocircuits (oligodendrocytes, axon/myelin integrity) and subsequently result in abnormal circuit connectivity affecting affective, cognitive and social functions (Steullet et al., 2010, Steullet et al., 2016). This hub can also be affected by environmental factors, the HPA-axis as well as neurotransmitters such as DA and 5-HT (Steullet et al., 2016).



**Figure 7:** Proposed central hub of schizophrenia (SCZ) – Simplified (Steullet et al., 2016). Abbreviations: 5-HT – serotonin, DA – dopamine, NMDA - N-methyl-D-aspartate glutamine.

Other findings linking oxidative stress to SCZ;

- SCZ patients present with reduced GSH levels (blood and plasma) compared to healthy subjects (Raffa et al., 2011, Micó et al., 2011). Imaging and post-mortem studies also found reduced GSH levels in the CSF, PFC (Do et al., 2000, Gawryluk et al., 2011) and striatum (Yao et al., 2006) of patients with SCZ. Raffa *et al.*, (2011) also found that the levels of GSH (blood and plasma) are positively correlated with the Scale for the Assessment of Positive Symptoms.
- Conversely, the GSSG levels in SCZ patients are elevated when compared to control groups (Raffa et al., 2011, Raffa et al., 2009).
- The total antioxidant status within the blood or serum are also significantly lower in first psychotic episode SCZ patients (Flatow et al., 2013).

### *Drug abuse and addiction*

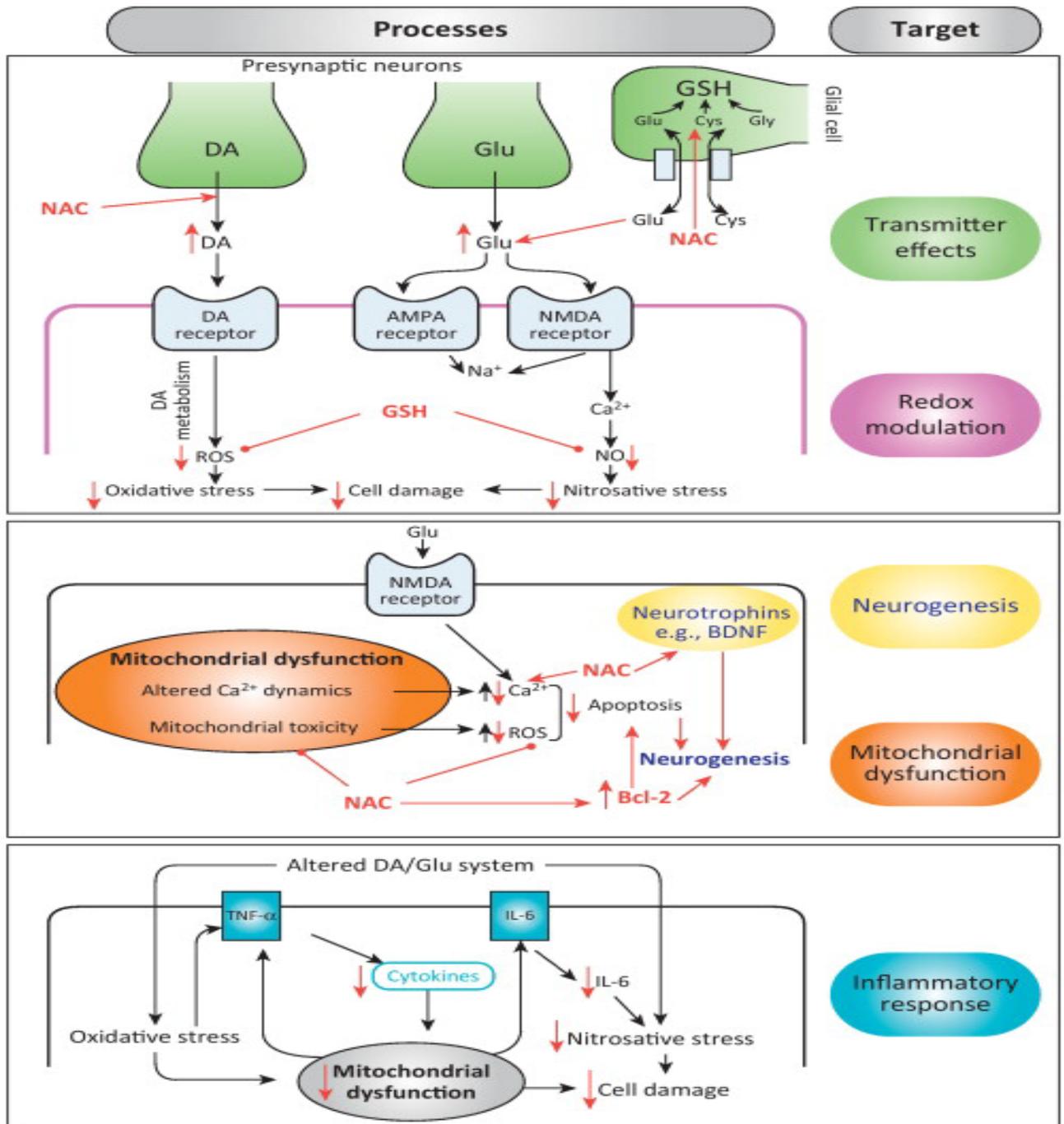
Findings of oxidative stress in drug abuse and addiction;

- Drugs of abuse induce a significant release of DA (Soleimani et al., 2016), which can induce oxidative stress after being oxidized through enzymatic- and non-enzymatic mechanisms (Cadet and Brannock, 1998).
- Previous pre-clinical studies found reduced plasma GSH levels after ethanol and opium exposure (Mohammadi et al., 2013).
- Pre-clinical studies have noted a reduction in GSH and GSSH levels after cocaine exposure within brain regions associated with the reward system such as the HPC (Muriach et al., 2010) as well as in the cerebellum (López-Pedrajas et al., 2015).
- A clinical study found increased levels of malondialdehyde (biomarker to determine lipid peroxidation and evaluating oxidative stress) in the serum of people abusing opioids and MA (Najafi et al., 2017).
- Amphetamine, cocaine, opioids and 3,4-methylenedioxy-methamphetamine induce oxidative stress in the CNS as reported by review literature and pre-clinical studies (Yamamoto and Bankson, 2005, Dietrich et al., 2005).

Oxidative stress is ever-present in many neuropsychiatric disorders, including Parkinson's disease (Chinta et al., 2006), SCZ (Wood et al., 2009, Steullet et al., 2016), Alzheimer's disease (Di Domenico et al., 2009), Huntington's disease (Klepac et al., 2007), depression (Brand et al., 2015), as well as addiction (Uys et al., 2011, Yamamoto et al., 2010). Considering this, treatment of the above with an antioxidant may be of significant value (Zhang and Yao, 2013, Pandya et al., 2013).

#### 1.10.1 NAC

NAC is considered a safe over-the-counter medication (Berk et al., 2013, LaRowe et al., 2006) used alone or adjunctive to treat a variety of conditions, including paracetamol poisoning (Green et al., 2013) and chronic obstructive pulmonary disease (Dekhuijzen and van Beurden, 2006). Other off-label uses include nephropathy (Quintavalle et al., 2013), atrial fibrillation (Liu et al., 2014), HIV (De Rosa et al., 2000), influenza A (Geiler et al., 2010) and more recently neuropsychiatric disorders (Dean et al., 2011, Berk et al., 2013). (see figure 8 for a visual representation), especially relating to neuroinflammation and oxidative stress.



**Figure 8:** N-acetylcysteine (NAC) pathophysiological targets – (Berk et al., 2013). Transmitter effects: NAC facilitate dopamine (DA) and glutamate (Glu) transmission. Redox modulation: NAC increase glutathione (GSH) which scavenge for reactive oxygen species (ROS) and nitrous oxide (NO). Neurogenesis: Neurogenesis can be directly- or indirectly promoted via NAC administration. Mitochondrial dysfunction: NAC restore mitochondrial dysfunction by altering calcium ( $Ca^{2+}$ ) dynamics. NAC can also reverse mitochondrial toxicity; this will lead to decreased ROS production via altered mitochondria metabolism. Inflammatory response: NAC reduce inflammatory responses by decreasing the cytokines (tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6)). Other abbreviations; Cys – cysteine, Gly – glycine,  $Na^{+}$  - Sodium, AMPA - 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, NMDA - *N*-methyl-D-aspartate (NMDA), BDNF - brain derived neurotrophic factor, Bcl-2 - B cell lymphoma 2

NAC possesses numerous mechanisms of actions which varies from being a mucolytic to influencing neurotransmission in the CNS (Dodd et al., 2008). One of its most prominent mechanisms involves the regulation of redox homeostasis (Dean et al., 2011). NAC can also alter the balance between anti- and pro-inflammatory cytokines, the latter a known contributor to neuropsychiatric diseases (Drexhage et al., 2010, Dean et al., 2011, Miller et al., 2013) and to be implicated in addiction (reviewed in (Beardsley and Hauser, 2014)).

Oral administration of NAC has the ability to penetrate the brain and thus to supplement/increase reduced GSH levels. This is better than administering GSH alone which is rapidly hydrolysed and has poor brain penetration (Dean et al., 2011). When NAC is administered the availability of cysteine (Cys) increases, this will subsequently result in the formation of GSH (thereby restoring GSH levels (Dean et al., 2011, Steullet et al., 2016), along with the other two peptides; GLU and glycine in the glial cells (Berk et al., 2013).

The alterations in Cys levels brought on by NAC can alter the DA and GLU neurotransmitter pathways (figure 8) (Himi et al., 2003, Janaky et al., 2007). Cys, a functional amino acid (Wu, 2009), regulates the neuronal intra- and extracellular exchange of GLU via the cystine-GLU antiporter which is found on all cell types (Dean et al., 2011, Baker et al., 2002). Cystine, the oxidised form of Cys (Banjac et al., 2008), is then taken up in exchange for GLU and released into the extracellular space (Dean et al., 2011). GLU subsequently stimulates the inhibitory metabotropic GLU autoreceptor, thus inhibiting GLU release (Moran et al., 2005). A pre-clinical study in rats also concluded that GSH could potentiate NMDA receptor response (Varga et al., 1997). Thus, elevated GLU levels induce oxidative stress which can be reversed or decreased with an antioxidant like NAC (Dean et al., 2011). NAC may also target DA-ergic systems; in a pre-clinical study where MA was repeatedly administered to monkeys, NAC restricted the lowering of DAT levels (Hashimoto et al., 2004). In another pre-clinical study using a model of SCZ (social isolation rearing (SIR)), NAC treatment reversed SIR-induced behavioural-, immune-inflammatory-, mitochondrial- and neurochemical (DA) alterations in rats (Möller et al., 2013). Furthermore, sub-chronic NAC treatment successfully reversed behavioural (recognition memory and anxiety-like social withdrawal behaviours), neurochemical (DA and NA), and oxidative stress-inflammatory (IL-10, TNF- $\alpha$ , lipid peroxidation and ROS) alterations induced by pre-natal inflammation, post-natal MA exposure and combined challenges (Swanepoel et al., 2018).

Dysfunctional DA and GLU metabolism will result in intracellular toxic metabolites such as nitrous oxide and ROS (Figure 8) (Berk et al., 2013). GSH will scavenge for these metabolites and by increasing GSH (via NAC) it will ultimately result in a reduced level of nitrosative- and oxidative stressors as well as cell damage in the brain (Berk et al., 2013). Altered neurotransmission (as described above) has the ability to activate inflammatory pathways which will subsequently cause mitochondrial dysfunction and cellular stress (Figure 8) (Berk et al., 2013). This inflammatory

response can be influenced via NAC by decreasing cytokine (IL-6 and TNF- $\alpha$ ) production (Berk et al., 2013). The mitochondrial dysfunction can also be restored by NAC by decreasing Ca<sup>2+</sup> intracellularly or modifying Ca<sup>2+</sup> dynamics within the mitochondria (Berk et al., 2013) (Figure 8). NAC administration can further promote neurogenesis either directly (increase in neuroprotective proteins (brain derived neurotrophic factor).) or indirectly (increasing antiapoptotic proteins (B cell lymphoma 2), thus, resulting in decreased apoptosis) (Figure 8) (Berk et al., 2013).

#### *NAC use in SCZ*

- NAC (1000 mg twice a day) reduced the negative symptoms experienced by SCZ patients (Berk et al., 2008, Lavoie et al., 2008). Another clinical study successfully used NAC (up to 2000 mg/day) as an augmentative strategy (Farokhnia et al., 2013).
- NAC (600 mg/day) improved positive and negative symptoms in SCZ patients (Bulut et al., 2009).

#### *NAC use in drug abuse and addiction*

- Pre-clinical and clinical studies in cannabis- (Gray et al., 2010, Gray et al., 2012), cocaine- (Kupchik et al., 2012, LaRowe et al., 2007), alcohol- (Morais-Silva et al., 2016) and nicotine addiction (Froeliger et al., 2015, Gipson et al., 2013) also found that NAC is successful in treating these addictions.
- Conversely, some clinical studies found that NAC (alone or in combination with another treatment) was ineffective on drug use or craving. This was found for MA (NAC 600-2400 mg/day + Naltrexone 50-200 mg/day) (Best et al., 2011), nicotine (NAC 2400 mg/day) (Knackstedt et al., 2009) and cocaine (NAC 1200, 1800 or 3600 mg/day) (Mardikian et al., 2007).
- Roberts-Wolfe and Kalivas (2015) reviewed numerous studies where NAC was found to improve drug abuse behaviour. Other pre-clinical- (Madayag et al., 2007, Chen et al., 2010) and clinical studies (Knackstedt et al., 2009) concluded that NAC is effective in treating addiction.

### **1.11 Animal models**

For decades animal models have been an essential part of neuropsychiatric research to help scientists understand and approach multiple psychiatric disorders (Kalivas et al., 2006). These models allowed the scientific community to study and evaluate the mechanisms of disease and helped develop and explore novel pharmacologic treatments (Krishnan et al., 2008). In 2006, van der Staay defined “*Animal models*”;

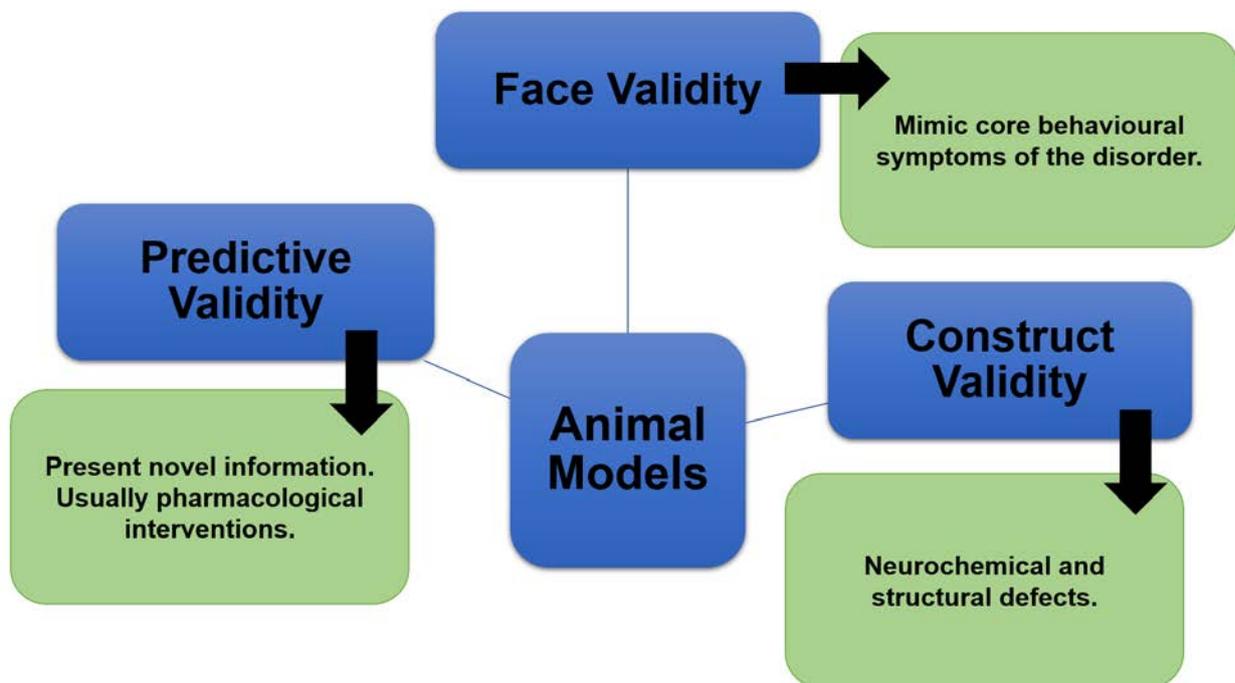
*“An animal model with biological and/or clinical relevance in the behavioural neurosciences is a living organism used to study brain–behaviour relations under controlled conditions, with the final goal to gain insight into, and to enable predictions about, these relations in humans and/or*

*a species other than the one studied, or in the same species under conditions different from those under which the study was performed.”*

*(van der Staay, 2006)*

Thomas R. Insel (2007) argued that even though animal models became such an important part of helping us understand emotions, learning, sensory perceptions as well as motor activity, these models still present with certain limitations, one being that psychiatric disorders occur due to various emotional, behavioural and cognition factors and not just singular excess or absence thereof (Insel, 2007). Even though Insel makes a valid argument, research in human beings themselves also has significant limitations, such as; the availability of human brains (Krishnan et al., 2008), ethical implications and experimental difficulties (Rubino and Parolaro, 2014).

In order for an animal model to be of use and have translational relevance, it should have construct, face and predictive validity to the clinical disorder being presented or modelled (Jones et al., 2011) (See figure 9 for a visual representation). Construct validity refers to the ability of the model to replicate the pathology as well as the theoretical neurobiological rational of the disorder (Jones et al., 2011). Face validity is the ability of the model to replicate symptom homology while predictive validity describes how accurately the model responds to known pharmacological treatments.



**Figure 9:** Animal model of psychiatric disorders – Adapted and simplified from Jones *et al.*, (2011). This schematic diagram is a representation of all domains needed for an animal model to be of translational relevance.

There are numerous possibilities when choosing an animal model of SCZ as well as drug reward and addiction. See table 1 for a short summary of all available models.

**Table 1:** Summary of available animal models applicable to psychotic disorders as well as drug abuse and addiction.

<b>Animal model</b>	<b>Addiction</b>	<b>Psychotic disorder</b>
Reinforcement model	DA-based positive reinforcement (reviewed by (H Johnston et al., 2016)).	
	Stress-based negative reinforcement (reviewed by (H Johnston et al., 2016)).	
	Giuliano model (Epstein and Kowalczyk, 2017)	
Self-administration model	Operant- and non-operant self-administration (reviewed by (Spanagel, 2017)).	
Genetically modified models	Reviewed by (Crabbe, 2016).	Reviewed by (van den Buuse, 2009)
KO models	Glucocorticoid receptor (Izawa et al., 2006)	DISC-1 (Jaaro-Peled, 2009)
		Neuregulin-1 & ErbB4 (Harrison and Law, 2006, Mei and Xiong, 2008)
		Dysbindin (Karlsgodt et al., 2011, Papaleo et al., 2012)
		Reelin (Krueger et al., 2006, Tueting et al., 2006)
Neurodevelopmental models	MIA model induced by LPS (Straley et al., 2017)	MIA induced by methylazoxymethanol (Moore et al., 2006, Lodge and Grace, 2009)
		MIA induced by LPS (Wischhof et al., 2015, Swanepoel et al., 2018)
		MIA induced by polyriboinosinic-polyribocytidylic acid (Poly I:C) (Wolff and Bilkey, 2010)
		SIR (Möller et al., 2011, Möller et al., 2013)
		Neonatal ventral hippocampal lesion (Lipska, 2004, Tseng et al., 2009)
Pharmacological models (drug induced models)		Amphetamine (Sarter et al., 2009, Featherstone et al., 2007).
		PCP (Neill et al., 2010, Mouri et al., 2007).

### 1.11.1 MIA

As stated before, early life stressors can contribute towards the susceptibility of developing a substance abuse disorder or addiction, as demonstrated by pre-clinical- (Bolton et al., 2018, Said et al., 2015) and clinical studies (Franzek et al., 2008, Dube et al., 2003). Also, important to note that an early life stressor can also contribute towards the development of a psychiatric disorder, such as SCZ (Möller et al., 2013, Wischhof et al., 2015), depression (Miller et al., 2009, Dallé and Mabandla, 2018), and an anxiety disorder (Wang et al., 2012). This study will focus on a pre-natal maternal infection as an early life stressor and the contribution thereof towards addictive- and psychosis-like bio-behavioural alterations presented in the off-spring later in life. Maternal infection during pregnancy is a noteworthy contributor to the development of SCZ (Straley et al., 2017, Borçoi et al., 2015), and therefore MIA in rodent dams presents with translational validity for application as an animal model for SCZ (Borçoi et al., 2015). This statement is further supported by evidence presented by Estes and McAllister (2016), stating that MIA mimics a disease-related risk factor (construct validity), exhibits an extensive range of symptoms related to the disease (face validity), while also being useful to determine efficacy of certain treatments (predictive validity). MIA has been used in numerous pre-clinical studies to investigate addictive- and psychosis-like bio-behavioural markers and behavioural response to treatment (Hava et al., 2006, Swanepoel et al., 2018).

Maternal infections in animal models typically involve immunological manipulation in pregnant dams at a certain gestational stage (Estes and McAllister, 2016). MIA can be achieved by administering a substance that mimics either a viral- (Poly I:C) or a bacterial infection (LPS) (Moller et al., 2015) to a pregnant rodent dam. In this study, we will only be focussing on LPS, an endotoxin from the Gram-negative bacterial group (Tenk et al., 2013). LPS binds to CD14 (a LPS receptor (Walter et al., 2006)) (Carpentier et al., 2005) that binds to the TLR<sub>4</sub> (Boksa, 2010, Feigenson et al., 2014). Such binding activates NFκB which results in increased cytokine (such as IL-1β, IL-6, and TNF-α) as well as chemokine (a chemotactic cytokine) production (Rivest, 2003). Other processes which occur after TLR<sub>4</sub> binding is activation of the complement cascade and the HPA axis as well as an increase in body temperature and inflammation (Tenk et al., 2013, Shanks et al., 1995, Boksa, 2010). These cytokines can cross the placenta to enter the foetus (Ashdown et al., 2006) where IL-6 and TNF-α for e.g. have been shown to activate microglia and astrocytes (Cai et al., 2000). This in turn induces the release of ROS and RNS with subsequent neuro-inflammation and cell death (Al-Amin et al., 2016). Therefore pre-natal inflammation is associated with oxidative stress (Al-Amin et al., 2016), as confirmed by numerous pre-clinical studies (Lanté et al., 2007, Ginsberg et al., 2012). The offspring from MIA models not only exhibit SCZ related behaviours (Meyer, 2014, Knuesel et al., 2014) but also induce various neurochemical alterations such as altered 5-HT and DA signalling (Knuesel et al., 2014, Reisinger

et al., 2015, Meyer, 2014) as well as abnormalities in the GABA (Hoftman et al., 2013, Schmidt and Mirnics, 2015) and GLU systems (Holloway et al., 2013, Zuckerman and Weiner, 2005).

MIA allows the study of neuro-psychiatric diseases in animals, such as SCZ (Brown and Derkits, 2009, Meyer and Feldon, 2009), autism spectrum disorder (Solek et al., 2017, Patterson, 2002) as well as depression and anxiety disorders (Depino, 2015, Masood et al., 2008). However, studies that link MIA and drug addiction are few (Straley et al., 2017, Borçoi et al., 2015). Thus, this study will provide more insight into this poorly studied topic.

### 1.11.2 Behavioural analysis

Pre-natal inflammation (using LPS) induces several behaviours in rats that are related to psychosis and cognitive deficits seen in humans with SCZ (Brown and Derkits, 2009, Boksa, 2010), as affirmed by numerous pre-clinical studies (Swanepoel et al., 2018, Allswede et al., 2016).

*Pre-pulse inhibition (PPI)*: PPI of the startle response is an assessment of sensorimotor gating which is compromised in numerous neuropsychiatric disorders (Swerdlow et al., 2008, Kumari et al., 2000). PPI presents the researcher with the ability to characterize core features of these disorders (i.e. SCZ) such as deficits in information processing as well as attention (Braff, 1993). Therefore, PPI cannot be characterized as an animal model of SCZ per se but rather a behavioural model that reflects sensorimotor gating deficits as seen in SCZ patients. This model presents with construct, face and predictive validity (Geyer et al., 2001).

Deficits in PPI (in rats) can be induced by developmental- or pharmacological manipulations, that is, by the stimulation of D<sub>2</sub> and 5-HT receptors as well as the blockage of NMDAR (for a comprehensive review see (Geyer et al., 2001)). PPI can be described as a reduced startle amplitude in response to a much stronger startling pulse or stimulus (Giakoumaki, 2012). It is important to highlight that the startling stimulus must be shortly preceded by a prepulse or pre-stimulus that is too weak itself to produce a measurable startle response (Giakoumaki, 2012). PPI will reflect sensorimotor gating, that is, the ability that an animal or human possess to collect appropriate sensory information through the surrounding environment (Tunstall et al., 2009) and filter out unnecessary input. This ability is crucial to understand external stimuli and to initiate appropriate behavioural responses (Tunstall et al., 2009). Deficits in sensorimotor gating culminate in a fragmentation of reality, as seen in illnesses like SCZ (Braff, 2010) or drug-induced psychotic episodes (Morales-Muñoz et al., 2017). PPI is therefore a behavioural test used to study the effects of MIA as well as psychotogenic drugs of abuse (Swanepoel et al., 2018, Meehan et al., 2017).

*Locomotor activity* : Locomotor activity is an essential trait in all animals where it is implicated (directly or indirectly) in nearly all behaviours (Martin, 2003). Locomotor activity reflects the action of the animal when placed in an immediate environment, and therefore it can be seen as a reflection of a decision-making process (Martin, 2003). Altered locomotor activity is evident in numerous disorders, including mood and anxiety disorders, SCZ, drug abuse and addiction (for a review see (Jones et al., 2011)). When the locomotor response becomes progressive and enhanced for a prolonged time it is referred to as locomotor sensitization, and provides insight into how certain factors (e.g. drugs of abuse) may induce chronic neuronal alterations. (Valjent et al., 2010, Robinson and Berridge, 1993). This sensitization can be induced after repeated exposure to addictive drugs of abuse and may persist up to a year in a drug-free period (Robinson and Berridge, 1993). In rodents this correlates with a heightened predisposition to self-administration of a psychoactive stimulant (Schenk and Partridge, 2000, Vezina et al., 2002), reinstatement of self-administration after it has been extinguished (Suto et al., 2004, De Vries et al., 1998), as well as compulsive drug-seeking behaviour (Vezina and Leyton, 2009, Robinson and Berridge, 1993). There is also evidence that connects motor activity to manic episodes, possibly due to patients presenting with increased activities which reflect DA dysregulation (Minassian et al., 2010).

*CPP*: CPP is one of the preferred models to study addiction in experimental animals (Tzschentke, 2007). CPP is considered a high-throughput method (Smith et al., 2016) that assesses reinforcing- and aversive effects of treatment (Lammel et al., 2012) as well as the rewarding properties of a drug (Smith et al., 2016). Numerous pre-clinical studies have used this method in addiction studies to evaluate the rewarding properties of known and putative drugs of abuse (Nelson et al., 2017, Faillace et al., 2018).

CPP makes use of Pavlovian conditioning (Huston et al., 2013, Schechter and Calcagnetti, 1993) wherein animals are taught to associate a reward- or an aversive state with specific contextual cues (Smith et al., 2016). The CPP apparatus consists of two distinct chambers that animals can access either through a doorway or through a smaller connecting chamber (Smith et al., 2016). The chambers differ from one another by either tactile (e.g., the texture of the floor), visual (e.g., the colour of the walls) and sometimes olfactory cues (Smith et al., 2016, Bardo and Bevins, 2000). The CPP test can be categorized into two designs, viz. 'biased-' and 'unbiased' (Smith et al., 2016). In a biased design the animal would typically show an innate preference towards one chamber and the aim would be to reverse this preference. In an unbiased design the animal does not have a preference towards one chamber and the aim is to create a preference for one chamber (Smith et al., 2016, Tzschentke, 1998). In this study, we will use a biased design as previously validated in our laboratory (Möller et al., 2018).

## Synopsis

Since HIV/AIDS became a worldwide epidemic with over 37 million victims (WHO, 2019), sufficient and effective treatment has become an inevitable intervention. HAART (which consists of several ARV classes) (Tse et al., 2015) ultimately resulted in significant improvements in the quality- and length of life of the patients (Hontelez et al., 2012). However, despite these benefits, HAART is not without fault. One particular ARV, EFV, soon presented with an array of problematic neuropsychiatric effects (Marinho et al., 2017) such as psychosis, hallucinations, delusions and paranoia (see review (Dalwadi et al., 2018)) that has eventually led to the misuse of this medicine, either alone (Inciardi et al., 2007) or in combination with other drugs of abuse (Larkan et al., 2010).

We are particularly interested in the sequelae of EFV misuse or abuse in individuals specifically in terms of developing an addictive- or SIPD which may ultimately result in SCZ (O'Connell et al., 2019). We would also like to investigate contributing factors towards these disorders, such as pre-natal inflammation (Straley et al., 2017, Scola and Duong, 2017) and its effect on behaviour as well as peripheral- and neurochemical bio-markers. Importantly, to our knowledge, no literature is available regarding an existing or even a possible treatment for these disorders (induced by EFV and/or exacerbated by a pre-natal stressor such as inflammation).

EFV has been known to affect several neurochemical (Gatch et al., 2013, Möller et al., 2018), immune- (O'Mahony et al., 2005), endocrine- (O'Mahony et al., 2005, Steensberg et al., 2003) and redox (Adjene et al., 2010) systems within the body. By exploring applicable bio-markers (c-Fos, GSH, PPP1R1B, DAT and CORT), we hope to provide new insight into the mechanism/s responsible for addictive-like and/or psychotic-like behaviour associated with EFV. Importantly, addiction and psychotic disorders (SCZ and SIPD) may have a bidirectional association or present with a dual-diagnosis (Gregg et al., 2007, Tohen et al., 1998).

In this study we will firstly investigate the ability of EFV to induce addictive-like- and psychotic-like behaviour (by measuring CPP, PPI and locomotor activity) as well as alterations in peripheral- and neurochemical bio-markers in rodents. Secondly, we will explore possible contributing factors towards these disorders and a probable bidirectional association (if any) between them. Lastly, we will explore a possible treatment option that centres around oxidative stress and that may alleviate the effects induced by EFV and/or pre-natal inflammation.

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## CHAPTER 3

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### MANUSCRIPT

Chapter 3 presents a concept article for submission to *Frontiers in Psychiatry*, a suitable and respected peer-reviewed scientific journal. This chapter was prepared according to the guidelines set out by the journal for authors (Addendum D). For a more in-depth description of all the guidelines, refer to <https://www.frontiersin.org/about/author-guidelines>.

The manuscript title, contributing authors and affiliations will be described on the following page. This is followed by an abstract and keywords as well as the main body of the manuscript according to the following structure: Introduction, Materials and Methods, Results and Discussion, Conclusion, Abbreviations, Acknowledgements, Author contributions, Conflict of interest and References. According to the author's guideline, all tables and figures should be added at the end of the manuscript. However, to benefit the reader all figures and tables were inserted in the text. *Frontiers* suggests the use of Times New Roman as a font as well as including page and line numbers to facilitate the review process. However, the font will be kept at Arial, no line numbers will be indicated and all heading- and page numbers will concur with that of the dissertation for aesthetic purposes. Before submission to *Frontiers in Psychiatry* the necessary changes will be made. *Frontiers* will apply the correct referencing style to the manuscript during typesetting. Therefore, the same referencing style was applied throughout the dissertation for aesthetic purposes.

C. Pieters undertook all behavioural and neurochemical studies, collected all data and undertook the statistical analysis. C. Pieters wrote the first draft of the manuscript. M. Möller supervised C. Pieters throughout the study and initiated and designed the study along with B.H. Harvey. M. Möller contributed towards the preparation of the final manuscript and B.H. Harvey provided critical revision of the manuscript. M. Möller and B.H. Harvey approved the final manuscript for submission.

All co-authors have granted permission for the submission of the article for the purpose of the MSc Pharmacology (Addendum E).

**Evaluating selected neuro-biological and behavioural properties of efavirenz in an inflammatory model of schizophrenia, and response to the antioxidant N-acetylcysteine.**

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## Abstract

**BACKGROUND:** Efavirenz (EFV) induces neuropsychiatric effects that contribute towards its abuse potential. Pre-natal inflammation and drug abuse later in life are pre-determinants of oxidative stress and the development of psychotic disorders. This study aimed to investigate addictive- and psychotic-like behaviours in rats after pre-natal exposure to bacterial lipopolysaccharide (LPS) and/or post-natal EFV exposure, and its response to N-acetylcysteine (NAC) treatment.

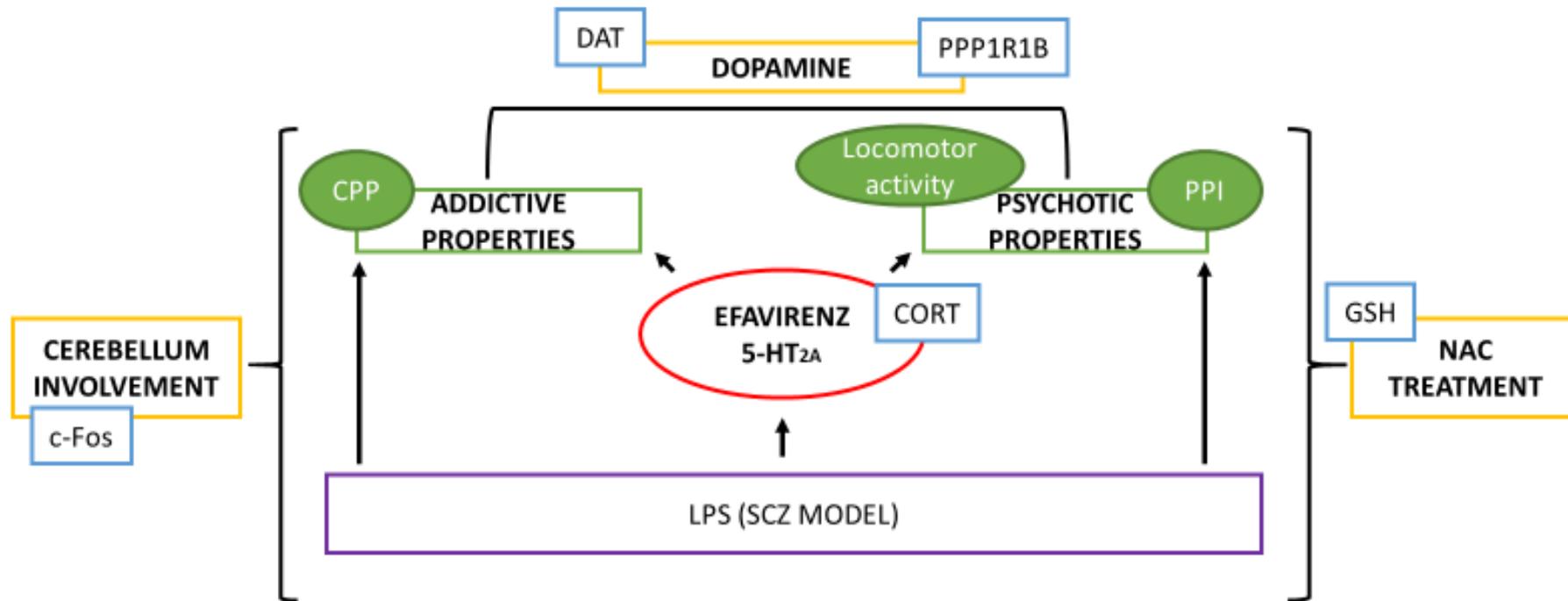
**METHODS:** Pregnant Sprague-Dawley rats (12/group) were exposed to either subcutaneous (SC) saline or 100 µg/kg LPS, with 96 male pups randomized into 8 groups (12/group). From post-natal day (PND) 48, exposure conditioning was performed on alternative days for six days with either olive oil (vehicle) or 5 mg/kg EFV and behavioural testing of conditioned place preference (CPP), locomotor and prepulse inhibition (PPI) testing. Thereafter, rats received either saline (vehicle) or 100 mg/kg NAC SC for 14 days. CPP and PPI analyses were repeated as per the pre-treatment methodology. On PND 73 rats were euthanised and plasma corticosterone (CORT) and glutathione (GSH), as well as cerebellar c-Fos and striatal and frontal-cortical, dopamine transporter (DAT) & phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B)), were analysed.

**RESULTS:** EFV (5 mg/kg) had no effect on CPP, locomotor activity or PPI compared to control groups. Pre-natal LPS (100 µg/kg) significantly induced decreased CPP, hyper-locomotor activity and %PPI deficits compared to control groups. These effects became more apparent in the LPS+EFV groups. The LPS+EFV significantly decreased striatal PPP1R1B and DAT and increased plasma CORT and cerebellar c-Fos compared to control groups. No differences were observed in frontal cortical PPP1R1B and DAT across all treatment groups. NAC (100 mg/kg) treatment failed to reverse any of the LPS, EFV or LPS+EFV associated alterations.

**CONCLUSION:** Maternal LPS induced psychotic-like behaviour in off-spring, but not addictive behaviour, while post-natal EFV did not induce addictive- or psychotic-like behaviour. The behavioural (locomotion and PPI), peripheral (CORT) and neurochemical (PPP1R1B) effects induced by LPS became more apparent after EFV exposure. Cerebellar immediate early gene activation (c-Fos) was only observed in the LPS+EFV group. NAC was not a viable treatment following EFV, LPS or LPS+EFV exposure.

**KEYWORDS:** Efavirenz, Lipopolysaccharide, N-acetylcysteine, Schizophrenia, Psychosis

Graphical abstract



## 1. Introduction

In 2019 the World Health Organization reported that 37.9 million individuals globally are infected with Human Immunodeficiency virus (HIV) and that by the end of 2018, 23.3 million were receiving antiretroviral (ARV) therapy (WHO, 2019). Efavirenz (EFV), classified as a non-nucleoside reverse transcriptase inhibitor, has been found to present with adverse neuropsychiatric effects, varying from irritability and nervousness to more severe neurological symptoms i.e. psychosis, anxiety, delusions and hallucinations (Apostolova et al., 2017). This ultimately led to patients discontinuing their ARV regimen (Blanch et al., 2001) or misusing the medication for the above-mentioned psychotropic effects (Mokwena, 2015, Grelotti et al., 2014). The sequelae of EFV abuse remains elusive and holds the possibility of inducing a substance-induced psychotic disorder (SIPD) or addiction.

A SIPD is considered a disorder where the patient experiences delusions and/or hallucinations for a short time period (approximately one month) upon acute intoxication or withdrawal of an addictive substance (APA, 2013). As addictive substances influence the mesolimbic dopamine (DA) pathway which underlies the development of psychotic symptoms (Fiorentini et al., 2011), these patients can subsequently develop schizophrenia (SCZ) (for a review see (O'Connell et al., 2019, Alderson et al., 2017)). SCZ is a severe neuropsychiatric disorder affecting 1% of the worldwide population (Berkovitch et al., 2018) where patients experience a combination of positive-(hallucinations and delusions), negative-(anhedonia, avolition and asociality) and cognitive symptoms (deficits in attention, memory and perception) (Migdalska-Richards and Mill, 2019).

Drug addiction is a disorder characterized by excessive drug taking and seeking despite the adverse consequences thereof (Zilverstand et al., 2018). Its behavioural proxy as well as clinical manifestations comprises of a chronic three-staged cycle, viz. intoxication, withdrawal and craving (Zilverstand et al., 2018). Previous literature has reported on the rampant abuse of EFV, either alone (Inciardi et al., 2007) or in a combination with other illicit substances (Mthembi et al., 2019, Dalwadi et al., 2018). This is quite concerning as this not only contributes towards another addiction crisis, but has significant potential to challenge the efficacy of HIV therapy due to ARV resistance (Grelotti et al., 2014, Larkan et al., 2010).

Previous literature reported on some main concerns as well as fundamental mechanisms of EFV which could ultimately contribute towards the development of a SIPD or addiction, as recently reviewed by Dalwadi and colleagues (2018). One of which is the ability of EFV to promote oxidative stress (Adjene et al., 2010) conceivably through altering antioxidants such as glutathione (GSH) and subsequently increase pro-inflammatory cytokines (O'Mahony et al., 2005), which may alter glucocorticoid (cortisol in humans, corticosterone (CORT) in rodents)

levels (Steensberg et al., 2003). Previous pre-clinical studies investigating SCZ found elevated levels of CORT within rats (Basta-Kaim et al., 2011a) which correlates with clinical findings reporting an increase in cortisol during the first psychotic episode (Mondelli et al., 2010, Pariante, 2008) as well as the acute phase of SCZ (Tandon et al., 1991). Furthermore, a recent review reported on how acute drug abuse in light or naïve users can increase CORT/cortisol within animals and humans (Wemm and Sinha, 2019) and it has been established previously that these drug-induced increases are connected to intoxication within healthy controls (Oswald et al., 2005). As mentioned earlier, EFV can promote oxidative stress, which occurs when there is an imbalance between the production of oxidants (i.e. reactive oxygen species (ROS) and reactive nitrogen species (RNS)) and the clearance thereof by antioxidants (Wood et al., 2009, Sayre et al., 2007). Oxidants can ultimately disrupt redox signalling and cause molecular damage (Sies and Jones, 2007, Moller et al., 2015). This process is evident in SCZ (Upthegrove and Khandaker, 2019, Bitanhirwe and Woo, 2011) and addiction (Beiser and Yaka, 2019, Kovacic and Cooksy, 2005). This is further supported by previous literature reporting on reduced levels of antioxidants such as GSH in both illnesses (Raffa et al., 2011, Flatow et al., 2013, Mohammadi et al., 2013, Uys et al., 2014).

DA transporters (DAT) regulate DA-ergic neurotransmission by clearing extraneuronal DA (Gether et al., 2006). The blocking thereof results in a profound increase in DA within the synaptic clefts (Ujike et al., 2003), especially in the nucleus accumbens (NAcc) responsible for inducing psychogenic- and reinforcing effects (Ujike, 2002). The euphoric- and reinforcing effects of psychostimulants such as methamphetamine and cocaine are attributed to their primary interaction with DAT (Mortensen and Amara, 2003), which in turn is dependent on the type of drug being abused (see (Liang et al., 2017, Leroy et al., 2012, Crits-Christoph et al., 2008) for further information). Furthermore considering clinical findings, striatal DAT availability is inversely correlated with the extent of hallucinatory experiences in patients with SCZ (Schmitt et al., 2006). It has been previously established that EFV blocks DAT (Gatch et al., 2013). Importantly, this was determined in vitro (Gatch et al., 2013) which is not necessarily an accurate predictor of a drug's behavioural- and psychostimulatory effects in vivo (Gether et al., 2006). However, a recent study in our laboratories found that EFV presented with dose-dependent addictive-like properties in vivo which could be attributed to elevated levels of DA, serotonin (5-HT) and noradrenaline in the striatum (Möller et al., 2018). Gatch and colleagues (2013) found that EFV presents with lysergic acid diethylamine (LSD)- like properties, as it has noteworthy 5-HT<sub>2A</sub> receptor stimulatory actions as well as induces a head twitch response in rats, a behavioural correlate of hallucinogenic potential in humans (González-Maeso et al., 2007). The stimulation of 5-HT<sub>2A</sub> ultimately results in DA release within the NAcc (Ham et al., 2017, Murase et al., 1993), a brain region implicated in both reward processing (Wouterlood et al., 2018) and some SCZ-related symptoms (Swerdlow et al., 1990, Swerdlow et al., 1992). DA in turn regulates DA-and-cyclic adenosine

monophosphate-regulated phosphoprotein (with a molecular weight of 32kD) (DARPP-32), also known as phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1) (Wang et al., 2017). DARPP-32 is a well-recognised sub-cellular biomarker for D<sub>1&2</sub> receptor mediated activity (Wang et al., 2017), and has been studied previously in addiction (Svenningsson et al., 2005, Zachariou et al., 2002) and SCZ (Albert et al., 2002, Feldcamp et al., 2008). DA receptors (D)<sub>1&2</sub> are involved in the modulation of c-Fos expression (Zornoza et al., 2005), an important immediate early gene involved in the regulation of synaptic plasticity, as studied in SCZ (Boyajyan et al., 2015) and addiction (Cruz et al., 2015). Moreover, recent research indicates that apart from the frontal cortex (FC) and striatal regions, the cerebellum is among the most affected brain regions in SCZ (Moberget et al., 2018) and addiction (Carta et al., 2019), with c-fos measurement in the cerebellum therefore a relevant neuronal activity marker (Carbo-Gas et al., 2014).

Maternal infection during pregnancy has been associated with neuropathological- and behavioural alterations in off-spring (Patterson, 2009, Zuckerman and Weiner, 2005), and more specifically to contribute towards the development of psychotic- or addictive disorders later in life. Indeed, simulated maternal infection in rodent dams, as induced by the bacterial endotoxin lipopolysaccharide (LPS) (Tenk et al., 2013) during pregnancy, is a widely used neurodevelopmental model of SCZ (Swanepoel et al., 2018, Straley et al., 2017, Brown and Derkits, 2009), presenting with face-, predictive- and construct validity.

Despite EFV being recognised as a potential intoxicating and addictive substance, no study has investigated a possible pharmacological treatment option for this condition. Based on the causal role of oxidative stress in the neuropsychiatric effects of EFV (Dalwadi et al., 2018) , we have investigated whether the antioxidant N-acetylcysteine (NAC) (a GSH precursor) may have therapeutic utility in this regard. This is especially relevant given NAC's ability to modulate redox homeostasis, inflammation and neurotransmission (Dean et al., 2011)) that in turn may contribute towards psychosis, SCZ and addiction (Möller et al., 2013a, Möller et al., 2013b, Grant et al., 2010, Sansone and Sansone, 2011, Slattery et al., 2015, Chen et al., 2016).

This study will therefore investigate the LPS model of psychosis in rats and whether EFV has the ability to induce addictive- and/or psychotic-like behaviour in rats alone and in the model, as determined by conditioned place preference (CPP), locomotor activity and prepulse inhibition (PPI) tests. Moreover, we will also study whether altered peripheral- (CORT and GSH) and neurochemical (PPP1R1B, DAT and c-Fos) bio-markers are implicated. This will ultimately reveal any contributory effects of EFV on LPS associated addictive- or psychotic-like behaviour. Lastly, we will investigate whether NAC may attenuate any bio-behavioural effects brought on by pre-natal inflammation and/or post-natal EFV.

## **2. Materials and methods**

### **2.1 Statement on ethics**

This study and manuscript were prepared and presented according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Kilkenny et al., 2010). This study was approved (Ethics approval number: NWU-00162-18-S5) by the AnimCare animal research committee (NHREC reg. no. AREC-130913-015) of the North West University (NWU). 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 at the NWU. The handling of animals, as well as the experiments, were according 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 Animals**

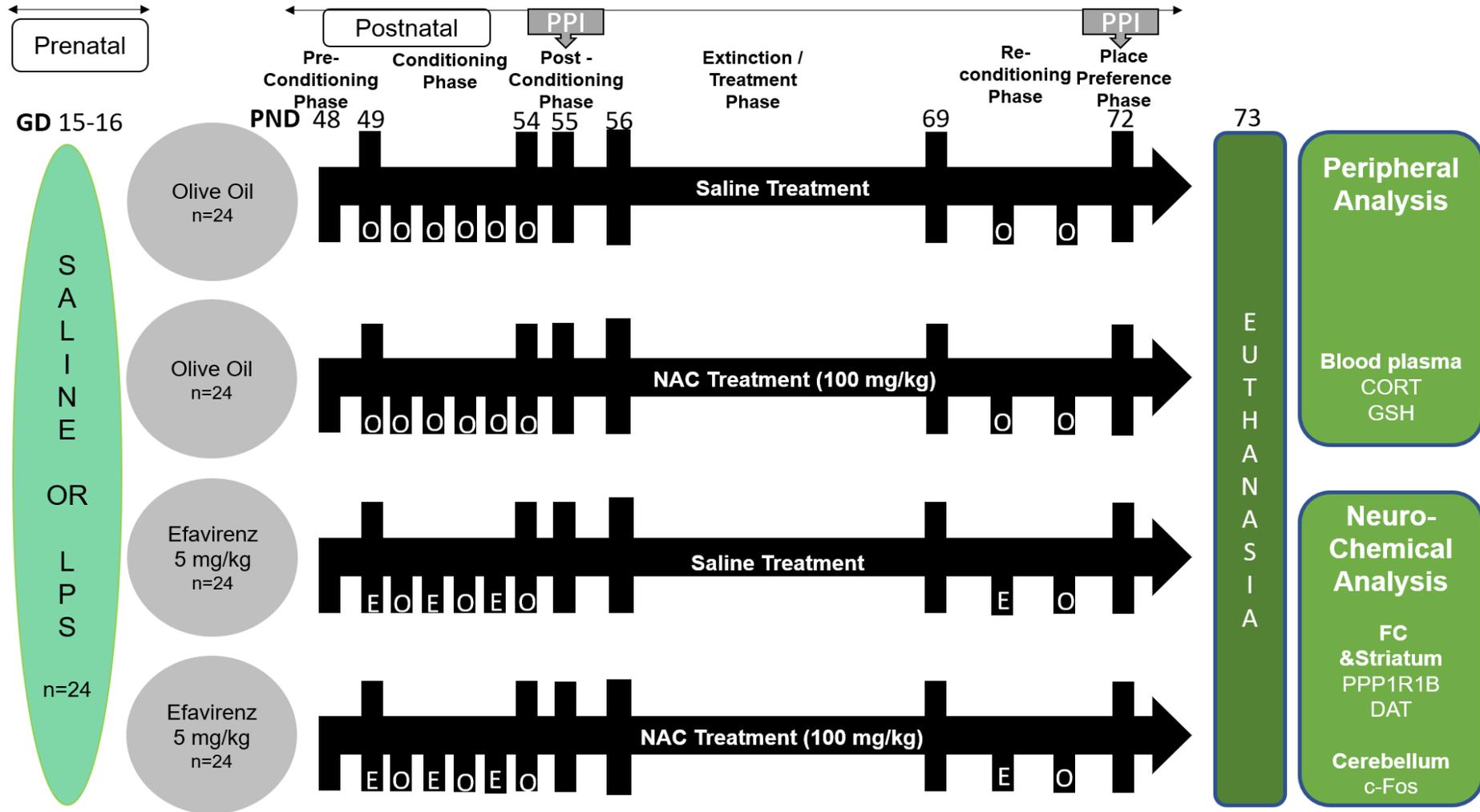
Pregnant Sprague-Dawley (SD) rats ( $n = 24$ ) were used for the pre-natal phase of the study and randomly divided into a control group (saline (SAL) exposure) ( $n = 12$ ) and a LPS exposure group ( $n = 12$ ). The numbers of female SD rats per SAL- and LPS exposure groups were to ensure sufficient male offspring for the next phase of the study. In the post-natal phase, the off-spring male SD rats were randomly divided into eight exposure groups each containing 12 rats per group. The rat quantities were based on a previous study (Swanepoel et al., 2018). A total of 120 SD rats (including, 24 pregnant dams and 96 male offspring) were used in this study.

The SD rats were housed under identical conditions: 2-3 rats per individual ventilated cage (230(h) x 380(w) x 380(l) mm) with corncob bedding and environmental enrichment (such as shredded paper for nesting purposes and a plastic (polyvinyl chloride) tube that can be used for playing, climbing, resting and hiding), temperature ( $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ), humidity ( $50 \pm 10\%$ ), white light (350-400 lux), 12h light/dark cycle (lights on 06h00 to 18h00) and food (pellets) and water were provided ad libitum (Mouton et al., 2016). The cages were changed three times a week with fresh corncob. Furthermore, the rats were monitored daily for any distress or discomfort (by making use of study-specific NWU Vivarium monitoring sheets), thereby ensuring the welfare of the animals.

### **2.3 Study design**

For a complete visual illustration of the study design, please see figure 1. The study consisted out of two cohorts, divided into a pre-natal- and post-natal phase of which the post-natal phase consisted of six sub-phases (Figure 1). During the pre-natal phase, pregnant SD rats ( $n = 24$ ) were divided into two groups; a SAL exposure group ( $n = 12$ ) and a LPS exposure group ( $n = 12$ ) (Figure 1). Exposure to SAL and LPS were on gestational day (GD) 15-16. Successful mating

and pregnancy were confirmed by the presence of a vaginal plug (Tain et al., 2018). The male offspring born from these two exposure groups remained with their biological mothers until they were weaned on post-natal day (PND) 21. A previous pre-clinical study demonstrated no protective effects of cross-fostering in a maternal immune activation (MIA) model (Meyer et al., 2006) and therefore it wasn't applied. Thereafter, the rats were randomly allocated to eight exposure groups (n = 12) and labelled according to their drug exposure regimen; 1. SAL-pharmaceutical grade olive oil (OO)-SAL, 2. SAL-OO-NAC, 3. SAL-EFV-SAL, 4. SAL-EFV-NAC, 5. LPS-OO-SAL, 6. LPS-OO-NAC, 7. LPS-EFV-SAL, 8. LPS-EFV-NAC. On PND 48 the post-natal experiments commenced and were divided into six sub-phases, in which the drug exposure and behavioural testing (CPP, locomotor activity & PPI) were applied throughout until PND 72 (Figure 1). The post-natal experimental design were based on and adapted from previous studies (Barbosa-Méndez et al., 2018, Möller et al., 2018). PND 73 marked the last day of the study as all animals were euthanised via decapitation (Figure 1). Brain tissue and trunk blood were collected and stored at -80°C until neuro-chemical- and peripheral analyses were performed.



**Figure 1:** Visual representation of the study design. Abbreviations: E- Efavirenz, O- Pharmaceutical grade olive oil, NAC – N-acetyl cysteine, FC – Frontal cortex, PPI- Pre-pulse inhibition, CCP- Conditioned place preference, DAT- dopamine transporters, CORT- Corticosterone, GSH- Glutathione, LPS- Lipopolysaccharide, PND- Post-natal day, GD- Gestational day.

## 2.4 Drugs and drug exposure protocol

During the pre-natal phase of the study, the exposure of pregnant dams to either saline or LPS occurred between 08h00 and 10h00 on GD 15-16 (Swanepoel et al., 2018). During this maternal exposure period (correlating with the late first trimester of human pregnancy (Aguilar-Valles and Luheshi, 2011)), the offspring tend to be more vulnerable towards developing psychotic-like behaviour later in life (Arsenault et al., 2014, Harvey and Boksa, 2012). During the post-natal phase of the study the rats were exposed to either OO or EFV and also received treatment with either NAC or SAL (depending on the groups they were divided into, as well as the specific sub-phase of the post-natal phase) between 08h00 and 10h00.

LPS from *Escherichia coli* (Sigma-Aldrich, Johannesburg, South Africa) was dissolved in isotonic SAL (0.9% sodium chloride (NaCl)) solution and administered subcutaneously (SC) to pregnant SD rats at a dosage of 100  $\mu$ g/kg (Swanepoel et al., 2018, Baharnoori et al., 2013). The vehicle (0.2ml/day) consisted of isotonic SAL (0.9% NaCl) solution. EFV (Aspen Pharmacare, South Africa) 5 mg/kg/day intraperitoneally was dissolved in a pharmaceutical grade OO solution (Möller et al., 2018). The vehicle (0.2ml/day) consisted of pharmaceutical grade OO (Möller et al., 2018). NAC (Sigma-Aldrich, Johannesburg, South Africa) (100 mg/kg SC) was dissolved in a isotonic SAL (0.9% NaCl) solution (Möller et al., 2013b). The constituted NAC solution were buffered with sodium hydroxide and 1 M glacial acetic acid (pH6) (Möller et al., 2013b). This dosage was based on similar pre-clinical addiction studies that obtained significant results using 100 mg/kg (Zhou and Kalivas, 2008, Moussawi et al., 2011, Reichel et al., 2011). Reichel and colleagues performed a dose-comparing study with NAC (60 mg/kg vs. 100 mg/kg) to assess relative effects on drug seeking behaviour, and found that 100 mg/kg was the most effective dose (Reichel et al., 2011). See table 1 for a detailed layout of the drug exposure and treatment protocol during the study.

**Table 1:** Drug exposure and treatment regimen

Post-natal day	Experimental phase	Experimental groups							
		SAL OO SAL	SAL OO NAC	SAL EFV SAL	SAL EFV NAC	LPS OO SAL	LPS OO NAC	LPS EFV SAL	LPS EFV NAC
48	Pre-Conditioning	None	None	None	None	None	None	None	None
49	Conditioning	OO	OO	EFV	EFV	OO	OO	EFV	EFV
50		OO	OO	OO	OO	OO	OO	OO	OO
51		OO	OO	EFV	EFV	OO	OO	EFV	EFV
52		OO	OO	OO	OO	OO	OO	OO	OO
53		OO	OO	EFV	EFV	OO	OO	EFV	EFV
54		OO	OO	OO	OO	OO	OO	OO	OO
55	Post-Conditioning	None	None	None	None	None	None	None	None
56 – 69	Extinction / Treatment	SAL	NAC	SAL	NAC	SAL	NAC	SAL	NAC

70	Re-Conditioning	OO	OO	EFV	EFV	OO	OO	EFV	EFV
71		OO							
72	Place Preference	None							

## 2.5 Body weight

The body weight of all experimental animals was determined each day (06h00 – 07h00) throughout the entire study (PND 48 – 72). This was done to calculate an accurate dose of the exposure drug and the amount of treatment drug needed for each individual animal and to ensure optimal growth in all groups.

## 2.6 Behavioural analysis

All behavioural tests were performed according to previously validated methods (Möller et al., 2013a, Akbarabadi et al., 2018, Barbosa-Méndez et al., 2018) during the light cycle (Möller et al., 2018). In the event where CPP and PPI tests were performed on the same day, the least stressful (CPP) test was performed first, followed by the most stressful test (PPI).

### CPP

CPP were performed throughout the six sub-phases (PND 48-72) of the post-natal period according to a method adapted from previous work (Barbosa-Méndez et al., 2018, Möller et al., 2018, Prus et al., 2009). The CPP test was performed in an apparatus containing three compartments made from plexiglass, each separated by guillotine doors. The two larger compartments (24 x 35 cm) were separated by a much smaller central compartment (15.5 x 19.5 cm), which was only used during the pre-, post-, re-conditioning and place preference testing phases (Mueller and Stewart, 2000, Barbosa-Méndez et al., 2018, Möller et al., 2018). The first large outer compartment had a wire mesh floor with black and white striped walls and the second large outer compartment had smooth plexiglass flooring with black walls (Möller et al., 2018). The small central compartment was neutral and had grey walls as well as grey flooring (Möller et al., 2018).

The CPP behavioural test were divided into six sub-phases: Phase 1: During the pre-conditioning phase the animal was placed in the small central compartment (Barbosa-Méndez et al., 2018) and left to wander about between the compartments for 20 minutes (Fourie et al., 2017), allowing it to habituate and develop a preference for one chamber (Prus et al., 2009). The time spent in each chamber was the indication of preference, and the least preferred chamber served as the chamber in which the drug (EFV) was administered to the animal (Prus et al., 2009). Phase 2: The conditioning phase involved the drug (EFV) being administered to the animal within the least-preferred chamber and a vehicle within the most-preferred chamber (Prus et al., 2009). The

animal was then left in the chamber for 20 min (Möller et al., 2018). Phase 3: In the post-conditioning phase the rats were returned to the CPP apparatus and allowed to wander about for 20 minutes (Bardo et al., 1995, Barbosa-Méndez et al., 2018, Fourie et al., 2017). The animals had access to all chambers as the guillotine doors were removed. The time which each animal spends in a specific chamber was calculated again and compared to the times calculated in the pre-conditioning phase (Bardo et al., 1995, Huston et al., 2013a). Phase 4: During the extinction / treatment phase the rats received a daily injection of either SAL or NAC (depending on the group they are allocated to). Phase 5: In the re-conditioning phase the animals received an EFV or pharmaceutical grade OO injection, returned to the applicable compartments and left to roam freely for 20 minutes (Barbosa-Méndez et al., 2018). Phase 6: During the place preference phase, the animals were returned to the apparatus in a drug-free state and were left to roam freely for 20 minutes. Once again, the animals had access to all chambers as the guillotine doors were removed. There after the time spent in each chamber was calculated (Barbosa-Méndez et al., 2018) and compared to the time spent in each chamber calculated in the pre-conditioning phase.

When considering the CPP paradigm results, an increase in time spent in the drug-paired compartment during the post-conditioning- and place preference phase with regards to the pre-conditioning phase was considered as evidence for CPP and a decrease in time would be evidence for conditioned place aversion (Gatch et al., 2013). Therefore, the following formula was used to calculate the difference in time spent in the drug-paired compartment;

CPP before NAC treatment: *Time spent in drug-paired compartment during post-conditioning phase (s) – Time spent in drug-paired compartment during pre-conditioning phase (s).*

CPP after NAC treatment: *Time spent in drug-paired compartment during place preference phase (s) – Time spent in drug-paired compartment during pre-conditioning phase (s).*

CPP behaviour was recorded under dim white light (30 lux) with a digital video camera. The CPP behaviour was blindly scored using EthoVision® XT software (Noldus Information Technology, Wageningen, Netherlands) as the time (seconds) spent in each compartment.

### Locomotor activity

Locomotor activity was scored during the post-conditioning- (PND 55) and place-preference phase (PND 72) when the animals were subjected to CPP testing. Therefore, the locomotor activity (distance moved) was measured within the CPP apparatus when the animals were free to move across all three compartments for 20 minutes. As mentioned above, this behaviour was recorded under dim white light (30 lux) with a digital video camera and all locomotor behaviour was blindly scored by EthoVision® XT software (Noldus Information Technology, Wageningen, Netherlands) as total distance moved (cm).

PPI

PPI was determined in the post-conditioning- (PND 55) and place-preference phase (PND 72). This test was performed in the same groups of animals after the CPP and locomotor activity testing, according to a previously published method (Möller et al., 2013a).

In short, each male SD rat was placed within a ventilated sound-attenuated startle chamber (SRLAB, San Diego Instruments, San Diego, USA) and habituated with 65dB background white noise for 5 minutes. This background white noise was present throughout the entire PPI test session. A 100 PPI test trials were performed with an average interval of 25 seconds. These PPI trials included a 20ms non-startling pre-pulse (72,76,80 or 84 dB) followed by a single 40ms startle stimulus (120 dB) 80ms later. PPI was performed in 4 blocks; Block 1: first ten pulse-alone stimuli, Block 2: 20 pulse-alone stimuli and pre-pulse stimuli, Block 3: 20 pulse-alone stimuli and pre-pulse stimuli and Block 4: last ten pulse-alone stimuli. The pulse-alone stimuli in these blocks served as a measure of the mean startle amplitude, which indicates the rat's habituation towards the re-occurring startling stimuli (Van den Buuse and Eikelis, 2001). The formula which is used to calculate PPI is as follows (Tunstall et al., 2009);

$$PPI = 100 - \left(100 \times \left(\frac{\text{mean}(\text{pulse alone}) - \text{mean}(\text{prepulse})}{\text{mean}(\text{pulse alone})}\right)\right)$$

After obtaining the %PPI data across all trials at 72, 76, 80 and 84 Db pre-pulses, an average %PPI was calculated (see formula below) and statistical analysis were performed accordingly.

$$AVERAGE \%PPI = \frac{\%PPI(72dB) + \%PPI(76dB) + \%PPI(80dB) + \%PPI(84dB)}{4}$$

**2.7 Peripheral and neurochemical analysis**Trunk blood collection

After decapitation, trunk blood was collected in pre-chilled tubes containing a heparin solution as an anti-coagulant. Thereafter, the plasma was centrifuged (15 minutes, 1000xg, 2-8°C) according to the method set out by Elabscience® for performing assays via an enzyme linked immunosorbent assay (ELISA) kit. The supernatant was collected and stored at -80°C (LAMB, NWU) until the day of analysis. On the day of analysis, the samples and kits were collected from the fridge and allowed to reach room temperature.

CORT

Rat plasma CORT was determined by means of the Rat CORT (corticosterone) ELISA kit (Catalog No: E-EL-R0269) 96T from Elabscience® USA, according to the manufacturer's

instructions. A BioTek FL600 Microplate Fluorescence Reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA) was used to determine the optical density (OD) at 450nm.

### GSH

Rat plasma GSH was determined by means of the GSH (Glutathione) ELISA kit (Catalog No: E-EL-0026) 96T from Elabscience® USA, according to the manufacturer's instructions. A BioTek FL600 Microplate Fluorescence Reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA) was used to determine the OD at 450nm.

### Brain dissection

After decapitation (PND 73), the brain was dissected into the FC, striatum and cerebellum. Stereotaxic co-ordinates were used to identify these brain regions as set out by (Paxinos and Watson, 1998). In short, the cerebellum was dissected first. Thereafter, the brain was dissected into the right- and left cerebral hemispheres, and the olfactory bulb removed. The FC was then dissected according to a method set out by (Toua et al., 2010), thereafter the remaining regions of the brain were placed on its ventral side and the striatum dissected out, using the external walls of the lateral ventricles as internal limits and the corpus callosum as external limit (Toua et al., 2010). The FC, striatum and cerebellum were snap frozen in liquid nitrogen and stored at -80°C (LAMB, NWU) until needed for further analysis. On the day before the assay, the brain tissue was prepared according to the appropriate methods (explained individually for each assay).

### c-Fos

The day before analysis, the cerebellum tissue was rinsed with ice-cold phosphate buffered solution (PBS) (pH 7.4), weighed and homogenised in 0.5ml PBS by sonication. The homogenates were centrifuged (2000-3000 RPM, 20 minutes), thereafter the supernatant was collected and stored at -80°C until the next day of analysis.

On the day of analysis, the samples and kits were collected from the fridge and allowed to reach room temperature. c-Fos protein expression in rat cerebellum were determined by means of the Rat c-Fos ELISA kit (Catalog No: E0046Ra) 96T from Bioassay Technology Laboratory China. A BioTek FL600 Microplate Fluorescence Reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA) was used to determine the optical OD at 450nm, according to the manufacturer's instructions.

### PPP1R1B

The day before analysis, the FC and striatal tissue was rinsed with ice-cold PBS (0.02M, pH 7.0-7.2) and weighed. To comply with the method set out by MyBioSource as well as the matter of

getting enough supernatant, two samples were pooled together. The tissues were then homogenised in PBS (tissue weight (mg): PBS ( $\mu$ l) volume = 1:1) by sonication. The homogenates were centrifuged (1500xg, 15 minutes), thereafter the supernatant was collected and stored at -80°C until the next day of analysis.

On the day of analysis, the samples and kits were collected from the fridge and allowed to reach room temperature. The PPP1R1B levels within the FC and striatum of rats were determined by means of the Rat Protein Phosphatase 1 Regulatory subunit 1B (PPP1R1B) ELISA kit (competitive ELISA) (Catalog No: MBS7245452) 96T from MyBioSource, according to the manufacturer's instructions. A BioTek FL600 Microplate Fluorescence Reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA) was used to determine the OD at 450nm.

### DAT

The day before analysis, the FC and striatum tissue was rinsed with ice-cold PBS (0.01M, pH 7.4), weighed and homogenised in PBS (tissue weight (g): PBS (ml) volume = 1:9) by sonication. The homogenates were centrifuged (5000xg, 5 minutes), thereafter the supernatant was collected and stored at -80°C until the next day of analysis.

On the day of analysis, the samples and kits were collected from the fridge and allowed to reach room temperature. The DAT levels within the FC and striatum of rats were determined by means of the Rat DAT (Dopamine Transporter) ELISA kit (Catalog No: E-EL-R0343) 96T from Elabscience® USA, according to the manufacturer's instructions. A BioTek FL600 Microplate Fluorescence Reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA) was used to determine the OD at 450nm.

## **2.8 Statistical analysis**

GraphPad Prism version 7 for Windows (GraphPad software, San Diego, USA) and SAS/STAT® software were used for the statistical analysis and graphical presentations, and all statistical analysis were done under the guidance of the Statistical Consultation Service of the NWU. The Shapiro Wilk test was used to test for normality. Body weight over the duration of the study for all eight exposure groups, were analysed by the Pearson's rank-order correlation test ( $r$ ). The strength of association deemed strong when  $r > 0.5$ . As a supportive measure, a one-way analysis of covariance (ANCOVA) with body weight on PND 48 (start of treatment period) as covariate was also performed. All behavioural tests analysed before NAC treatment were done by using a two-way analysis of variance (ANOVA) with pre-natal exposure (saline or LPS) and drug exposure (EFV or OO) being the two variables. Behavioural-, peripheral and neurochemical tests performed after NAC treatment were analysed by a three-way ANOVA. The variables being pre-natal

exposure (SAL or LPS), drug exposure (EFV or OO) and treatment (NAC or SAL). In the event of a three-way ANOVA not showing a significant three-way effect, a two-way ANOVA was applied to investigate the main effects further. This was also applied to the two-way ANOVAs, whereas we performed an unpaired student's t-test if deemed necessary. Tukey's multiple comparisons post hoc testing were applied when appropriate in all ANOVAs. In all cases, data are expressed as the mean  $\pm$  standard error of the mean (SEM), where a p-value of  $<0.05$  or Cohen's d-value of  $>0.8$  is considered as statistically significant.

### 3. Results

#### 3.1 Body weight

There were strong correlations between age and body weight for all test groups ( $r > 0.9$ ), yet, no significant differences were observed between the slopes of body weight regression lines for any of the treatment groups [ $F(7, 2382) = 1.14, p = 0.3343$ ] (data not shown). In support, a one-way ANCOVA was performed to determine any mean differences of body weight on PND72 (i.e. end of treatment period) between the different treatment groups, using the weight of PND48 (start of treatment period) as covariate. No significant differences [ $F(7.85) = 1.746, p = 0.109$ ] were observed between any of the test groups for body weight on PND72 (data not shown).

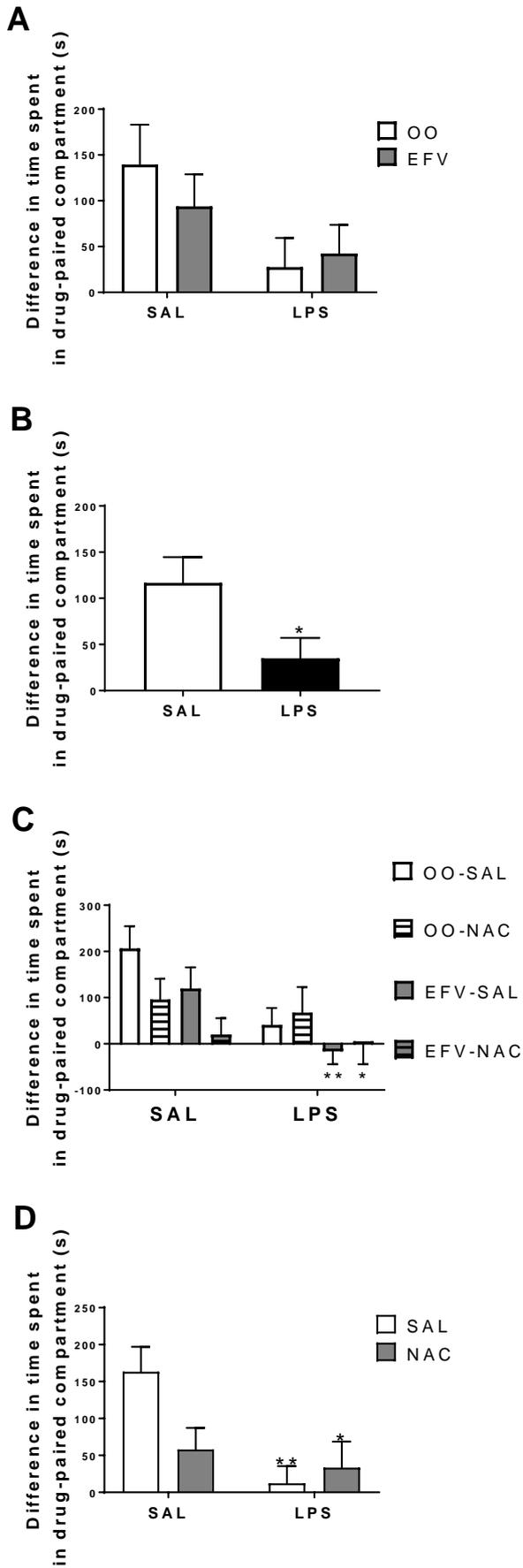
#### 3.2 Behavioural analysis

##### **CPP**

CPP before NAC treatment: Two-way ANOVA revealed a significant effect of pre-natal exposure [ $F(1.92) = 5.221, p = 0.0246$ ], but no significant two-way interaction between pre-natal exposure x drug exposure [ $F(1.92) = 0.7125, p = 0.4008$ ] as well as no significant effect of drug exposure [ $F(1.92) = 0.1847, p = 0.6684$ ]. Tukey's multiple comparisons post-hoc analysis also showed no significant differences between the groups (Figure 2A). Unpaired student's t-test revealed a significant decrease in time spent in drug-paired compartments in the pre-natal LPS versus SAL group, irrespective of drug exposure ( $p = 0.0238$ ) (Figure 2B).

CPP after NAC treatment: Three-way ANOVA revealed a significant two-way interaction between pre-natal exposure x treatment [ $F(1.88) = 4.332, p = 0.0403$ ] along with a significant effect of pre-natal exposure [ $F(1.88) = 8.351, p = 0.0049$ ] and drug exposure [ $F(1.88) = 5.592, p = 0.0202$ ]. No significant three-way interaction between pre-natal exposure x drug exposure x treatment [ $F(1.88) = 0.03238, p = 0.8576$ ] or two-way interactions between pre-natal exposure x drug exposure [ $F(1.88) = 0.09236, p = 0.7619$ ], drug exposure x treatment [ $F(1.88) = 2.344e-005, p = 0.9961$ ] or effect of treatment [ $F(1.88) = 1.914, p = 0.1700$ ], were observed. Tukey's multiple comparisons post-hoc analysis indicated a significant decrease in time spent in drug-paired compartments in the LPS-EFV-SAL group ( $p = 0.0095$ ) and the LPS-EFV-NAC group ( $p = 0.0212$ ) compared to

the SAL-OO-SAL group (Figure 2C). Two-way ANOVA revealed a significant two-way interaction between pre-natal exposure x treatment [ $F(1.92) = 4.253$ ,  $p = 0.0420$ ] and a significant effect of pre-natal exposure [ $F(1.92) = 8.198$ ,  $p = 0.0052$ ]. No significant effect of treatment [ $F(1.92) = 1.879$ ,  $p = 0.1738$ ] was observed. Tukey's multiple comparisons post-hoc analysis indicated a significant decrease in time spent in drug-paired compartments in the LPS-SAL group ( $p = 0.0042$ ) and LPS-NAC group ( $p = 0.0183$ ) compared to the SAL-SAL group (Figure 2D).

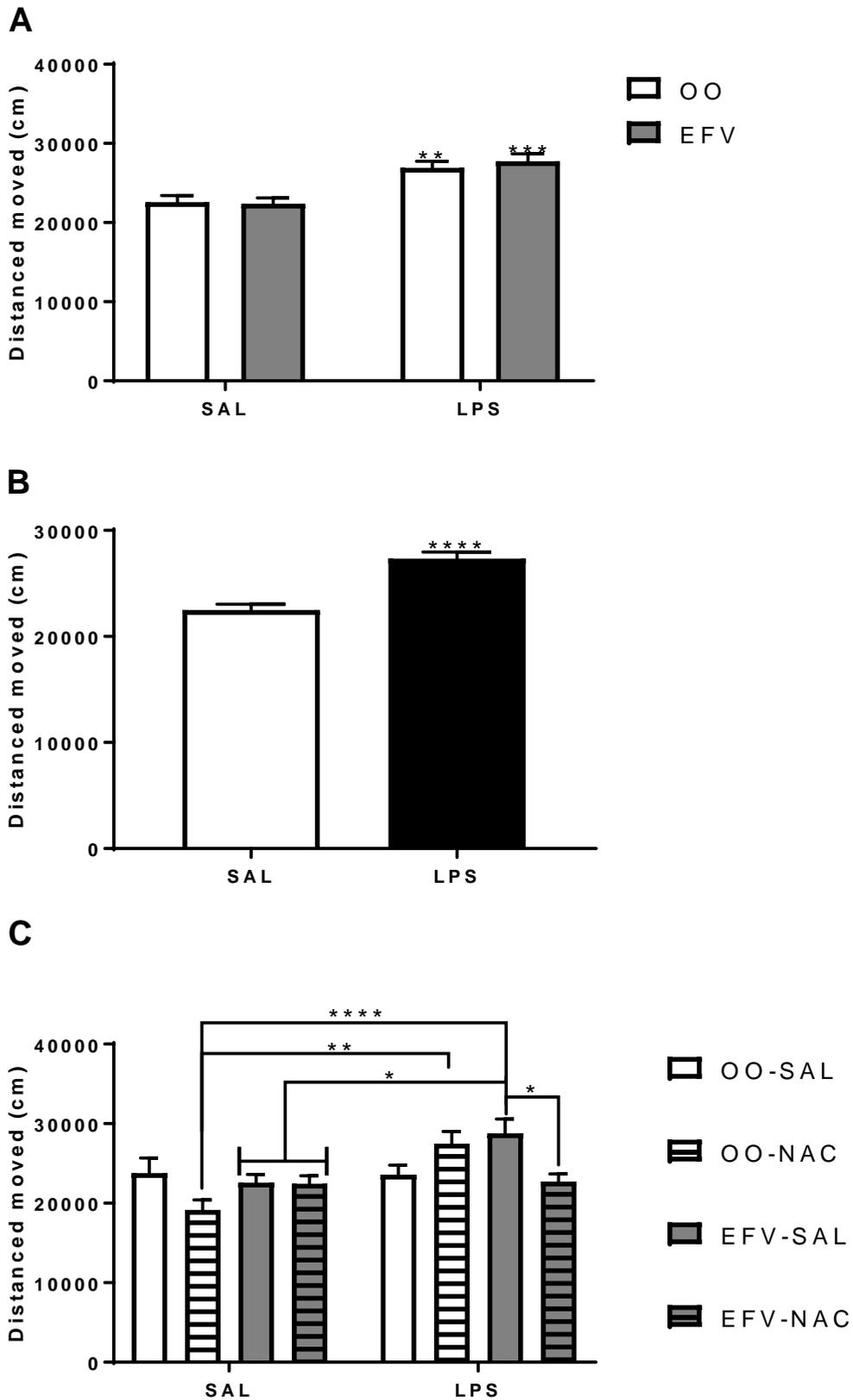


**Figure 2 A-D.** Conditioned place preference (CPP): **A** (CPP before N-acetylcysteine (NAC) treatment) – Place preference produced by pre-natal lipopolysaccharide (LPS) exposure, post-natal efavirenz (EFV) exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 24 animals per group (two-way ANOVA, Tukey's multiple comparisons test). **B** (CPP before NAC treatment) - Place preference produced by pre-natal LPS and saline (SAL) exposure. Data are expressed as mean  $\pm$  SEM of 48 animals per group. \* $p < 0.05$  vs. SAL (Unpaired student's t-test). **C** (CPP after NAC treatment) - Place preference produced after NAC treatment and previous post-natal EFV exposure, pre-natal LPS exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \* $p < 0.05$ , \*\* $p < 0.01$  vs. SAL-OO-SAL (three-way ANOVA, Tukey's multiple comparisons test). **D** (CPP after NAC treatment) - Place preference produced by NAC treatment and previous pre-natal exposure to LPS. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \* $p < 0.05$ , \*\* $p < 0.01$  vs. SAL-SAL (two-way ANOVA, Tukey's multiple comparisons test).

### ***Locomotor activity measured in the CPP.***

Locomotor activity before NAC treatment: Two-way ANOVA revealed a significant effect of pre-natal exposure [ $F(1.92) = 33.67$ ,  $p < 0.0001$ ], but no significant two-way interaction between pre-natal exposure x drug exposure [ $F(1.92) = 0.3746$ ,  $p = 0.5420$ ] and no significant effect of drug exposure [ $F(1.92) = 0.1568$ ,  $p = 0.6931$ ]. Tukey's multiple comparisons post-hoc analysis indicated a significant increase in distance moved in the LPS-OO group ( $p = 0.0023$ ) and LPS-EFV group ( $p = 0.0002$ ) compared to SAL-OO group as well as the LPS-OO group ( $p = 0.0013$ ) and LPS-EFV group ( $p = 0.0001$ ) compared to the SAL-EFV group (Figure 3A). Unpaired student's t-test revealed a significant increase in distance moved in the animals exposed to pre-natal LPS versus that of the SAL group ( $p < 0.0001$ ) (Figure 3B).

Locomotor activity after NAC treatment: Three-way ANOVA revealed a significant three-way interaction between pre-natal exposure x drug exposure x treatment [ $F(1.88) = 14.01$ ,  $p = 0.0003$ ] and a significant effect of pre-natal exposure [ $F(1.88) = 14.3$ ,  $p = 0.0003$ ]. No significant two-way interactions between pre-natal exposure x drug exposure [ $F(1.88) = 0.1847$ ,  $p = 0.6684$ ], drug exposure x treatment [ $F(1.88) = 2.006$ ,  $p = 0.1602$ ], pre-natal exposure x treatment [ $F(1.88) = 0.4435$ ,  $p = 0.5072$ ] or effects of drug exposure [ $F(1.88) = 0.4064$ ,  $p = 0.5255$ ] and treatment [ $F(1.88) = 3.224$ ,  $p = 0.0760$ ], were observed. Tukey's multiple comparisons post-hoc analysis indicated a significant increase in distance moved in the LPS-OO-NAC group ( $p = 0.0011$ ) and LPS-EFV-SAL group ( $p < 0.0001$ ) compared to the SAL-OO-NAC group (Figure 3C). A significant increase in distance moved in the LPS-EFV-SAL group ( $p = 0.0371$ ) compared to the SAL-EFV-SAL group and in the LPS-EFV-SAL group ( $p = 0.0306$ ) compared to the SAL-EFV-NAC group were observed (Figure 3C). Also, a significant increase in distance moved was observed in the LPS-EFV-NAC group ( $p = 0.0450$ ) compared to the LPS-EFV-SAL group (Figure 3C).



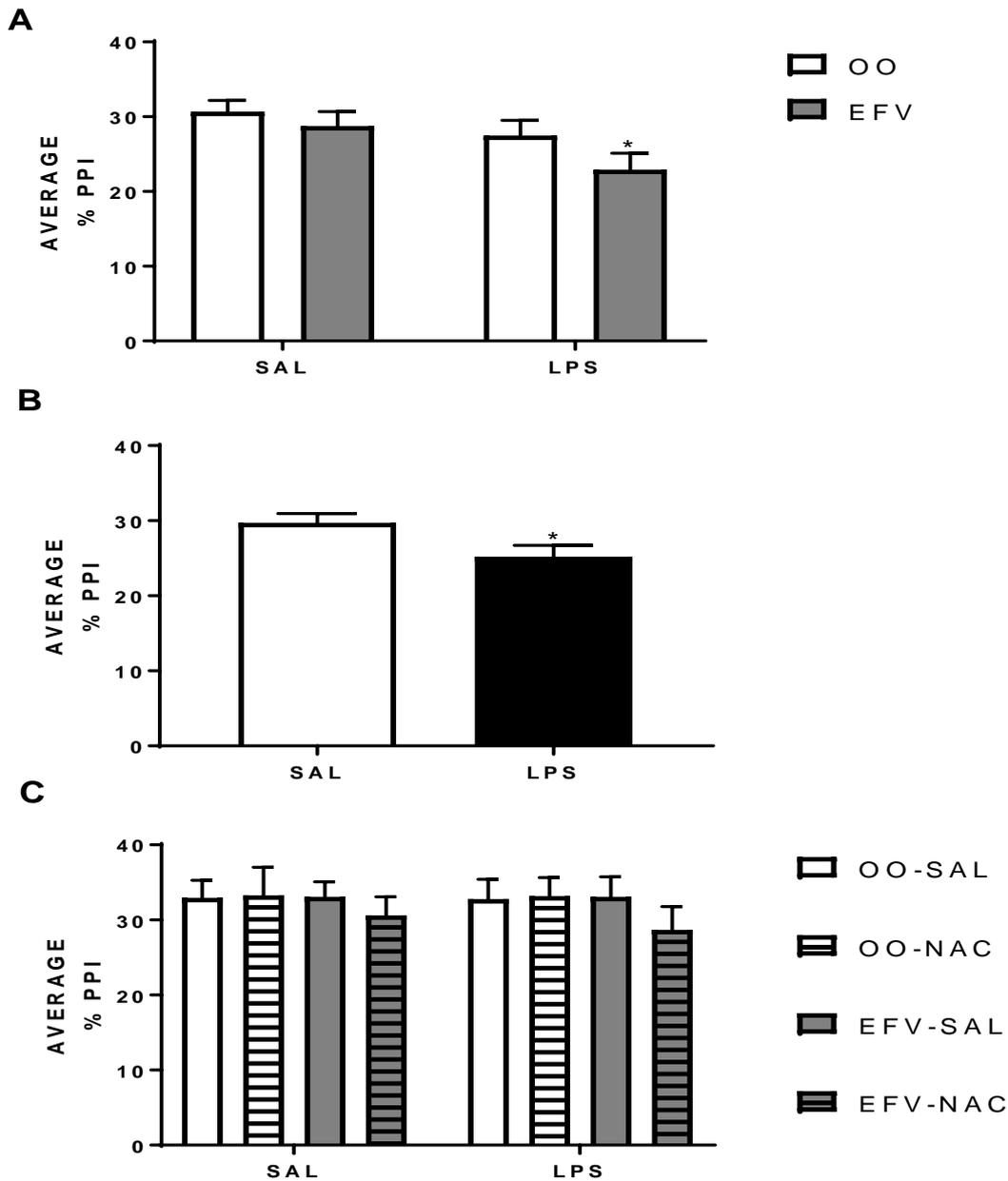
**Figure 3 A-C:** Locomotor activity. **A** (Locomotor activity before N-acetylcysteine (NAC) treatment) - Locomotor activity produced by pre-natal lipopolysaccharide (LPS) exposure, post-natal efavirenz (EFV) exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 24 animals per group.

\*\*p < 0.001, \*\*\*p < 0.0001 vs. saline (SAL) groups (two-way ANOVA, Tukey's multiple comparisons test). **B** (Locomotor activity before NAC treatment) - Locomotor activity induced by pre-natal LPS and SAL exposure. Data are expressed as mean  $\pm$  SEM of 48 animals per group. \*\*\*\*p < 0.0001 vs. SAL (Unpaired student's t-test). **C** (Locomotor activity after NAC treatment) - Locomotor activity produced after NAC treatment and previous pre-natal LPS exposure, post-natal EFV exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \*p < 0.05 vs. LPS-EFV-SAL. \*\*p < 0.01 vs. LPS-OO-NAC. \*\*\*\*p < 0.0001 vs. LPS-EFV-SAL (three-way ANOVA, Tukey's multiple comparisons test).

### **Average %PPI of acoustic startle**

Average %PPI before NAC treatment: Two-way ANOVA revealed a significant effect of pre-natal exposure [F(1.92) = 5.391, p = 0.0225], but no significant two-way interaction between pre-natal exposure x drug exposure [F(1.92) = 0.4553, p = 0.5015] and no significant effect of drug exposure [F(1.92) = 2.806, p = 0.0973]. Tukey's multiple comparisons post-hoc analysis indicated a significant decrease in average %PPI in the LPS-EFV group (p = 0.0290) compared to the SAL-OO group (Figure 4A). Unpaired student's t-test revealed a significantly decreased average %PPI in pre-natal LPS exposed animals versus the SAL group (p = 0.0233) (Figure 4B).

Average %PPI after NAC treatment: Three-way ANOVA revealed no significant three-way interaction between pre-natal exposure x drug exposure x treatment [F(1.88) = 0.06641, p = 0.7972] or two-way interactions between pre-natal exposure x drug exposure [F(1.88) = 0.04565, p = 0.8313], drug exposure x treatment [F(1.88) = 0.9672, p = 0.3281], pre-natal exposure x treatment [F(1.88) = 0.05794, p = 0.8103] as well as effects of pre-natal exposure [F(1.88) = 0.08188, p = 0.7754], drug exposure [F(1.88) = 0.764, p = 0.3844] and treatment [F(1.88) = 0.6279, p = 0.4303]. Tukey's multiple comparisons post-hoc analysis indicated no significant differences between the groups (Figure 4C).

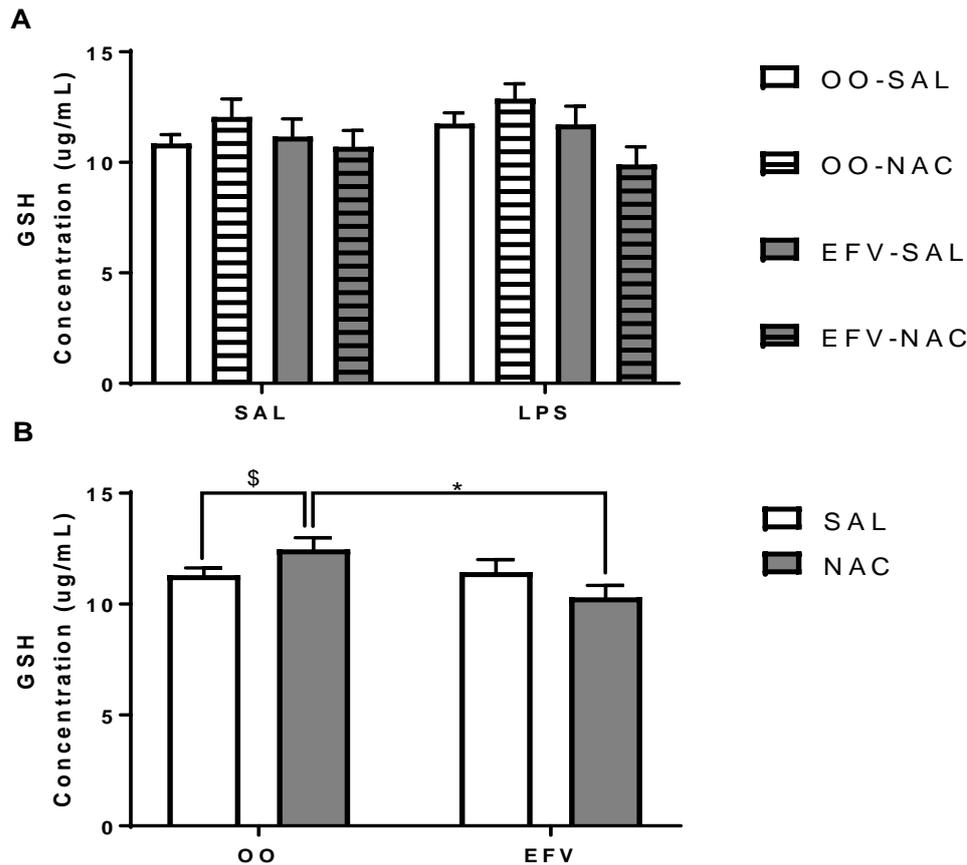


**Figure 4 A-C.** Average %pre-pulse inhibition (PPI) **A** (Average %PPI before N-acetylcysteine (NAC) treatment): Average %PPI produced by pre-natal lipopolysaccharide (LPS) exposure, post-natal efavirenz (EFV) exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \* $p < 0.05$  vs. saline-olive oil (SAL-OO) (two-way ANOVA, Tukey's multiple comparisons test). **B** (Average %PPI before NAC treatment): Average %PPI produced by pre-natal LPS and SAL exposure. Data are expressed as mean  $\pm$  SEM of 48 animals per group. \* $p < 0.05$  vs. SAL (Unpaired student's t-test). **C** (Average %PPI after NAC treatment): Average %PPI produced after NAC treatment and previous pre-natal LPS exposure, post-natal EFV exposure and a combination of EFV and LPS. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test).

### 3.3 Peripheral analysis

#### **GSH**

Three-way ANOVA revealed a significant two-way interaction between drug exposure x treatment [ $F(1.88) = 5.284$ ,  $p = 0.0239$ ] and a significant effect of drug exposure [ $F(1.88) = 4.117$ ,  $p = 0.0455$ ]. No significant three-way interaction between pre-natal exposure x drug exposure x treatment [ $F(1.88) = 0.3987$ ,  $p = 0.5294$ ], two-way interactions between pre-natal exposure x drug exposure [ $F(1.88) = 0.9722$ ,  $p = 0.0239$ ], pre-natal exposure x treatment [ $F(1.88) = 0.5143$ ,  $p = 0.4752$ ] or effects of pre-natal exposure [ $F(1.88) = 0.5446$ ,  $p = 0.4625$ ] and treatment [ $F(1.88) = 0.000603$ ,  $p = 0.9805$ ], were observed. Tukey's multiple comparisons post-hoc analysis indicated no significant differences between the groups (Figure 5A). Two-way ANOVA revealed a significant two-way interaction between drug exposure x treatment [ $F(1.92) = 5.376$ ,  $p = 0.0226$ ] and a significant effect of drug exposure [ $F(1.92) = 4.189$ ,  $p = 0.0436$ ]. No significant effect of treatment [ $F(1.92) = 0.0006135$ ,  $p = 0.9803$ ] was observed. Tukey's multiple comparisons post-hoc analysis indicated a significant decrease in GSH concentration in the EFV-NAC group ( $p = 0.0140$ ) compared to the OO-NAC group (Figure 5B). A medium practical effect size of an increase in GSH was observed in the OO-NAC (Cohen's d-value = 0.57) ( $p = 0.3523$ ) in comparison to the control (SAL-OO-SAL) group (Figure 5B).

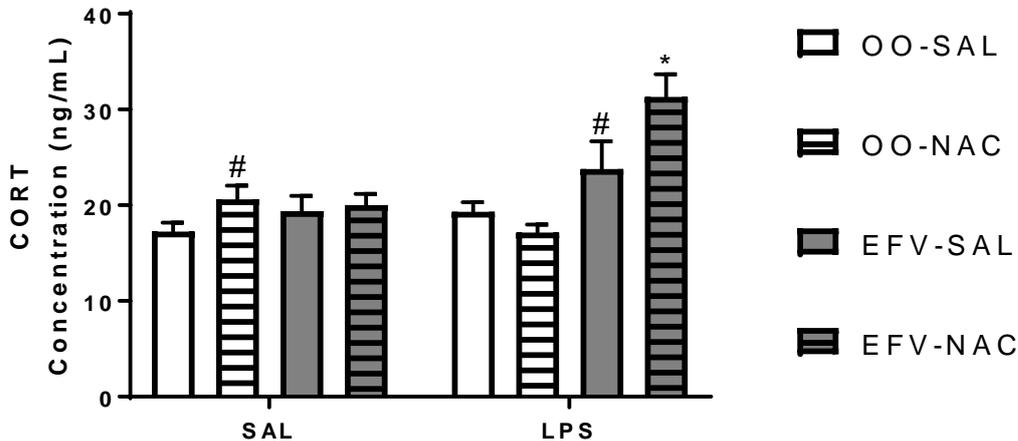


**Figure 5A-B:** Plasma glutathione (GSH): **A** GSH concentration levels in the plasma of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test). **B** Plasma GSH: GSH concentration levels in the plasma of rats after post-natal EFV exposure as well as NAC treatment. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \* $p < 0.05$  vs. EFV-NAC (two-way ANOVA, Tukey's multiple comparisons test). \$ $d > 0.5$ , vs. the olive oil-saline (OO-SAL) group (Cohen's d-value).

### CORT

Three-way ANOVA revealed a significant three-way interaction between pre-natal exposure x drug exposure x treatment [ $F(1.88) = 6.83$ ,  $p = 0.0105$ ], a significant two-way interaction between pre-natal exposure x drug exposure [ $F(1.88) = 12.83$ ,  $p = 0.0006$ ] and significant effects of pre-natal exposure [ $F(1.88) = 9.015$ ,  $p = 0.0035$ ] and drug exposure [ $F(1.88) = 17.79$ ,  $p < 0.0001$ ]. No significant two-way interactions between drug exposure x treatment [ $F(1.88) = 2.116$ ,  $p = 0.1493$ ], pre-natal exposure x treatment [ $F(1.88) = 0.07804$ ,  $p = 0.7806$ ], or an effect of treatment [ $F(1.88) = 3.843$ ,  $p = 0.0531$ ], were observed. Tukey's multiple comparisons post-hoc analysis indicated a significant increase in CORT plasma concentration in the LPS-EFV-NAC group compared to all the other groups [SAL-OO-SAL ( $p < 0.0001$ ), SAL-OO-NAC ( $p = 0.0006$ ), SAL-EFV-SAL ( $p < 0.0001$ ), SAL-EFV-NAC ( $p = 0.0002$ ), LPS-OO-SAL ( $p < 0.0001$ ), LPS-OO-NAC ( $p < 0.0001$ ),

and LPS-EFV-SAL ( $p = 0.0438$ )] (Figure 6). A large practical effect size of an increase in CORT was observed in the SAL-OO-NAC (Cohen's  $d$ -value = 0.81) ( $p = 0.8457$ ) and the LPS-EFV-SAL (Cohen's  $d$ -value = 0.88) ( $p = 0.1237$ ) groups in comparison to the control (SAL-OO-SAL) group (Figure 6).



**Figure 6.** Plasma corticosterone (CORT): CORT concentration levels in the plasma of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \* $p < 0.05$ , vs. all the other groups (three-way ANOVA, Tukey's multiple comparisons test). # $d > 0.8$ , vs. the saline-olive oil-saline (SAL-OO-SAL) group (Cohen's  $d$ -value).

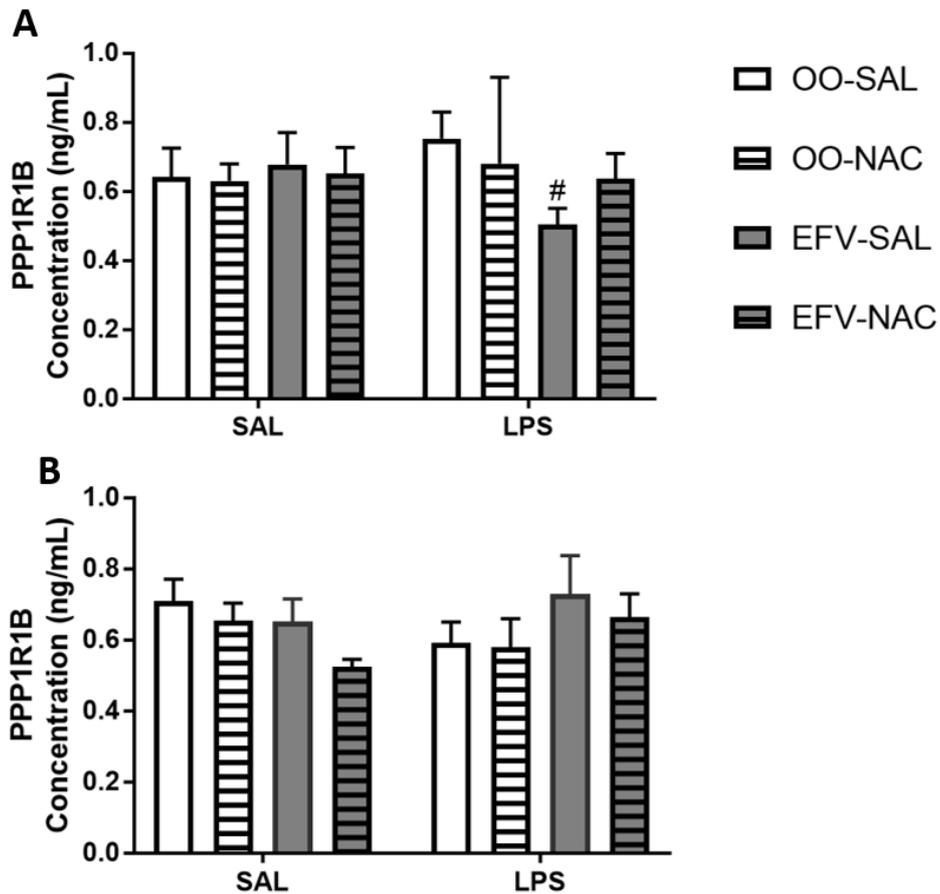
### 3.4 Neurochemical analysis

#### PPP1R1B

Striatal PPP1R1B: Three-way ANOVA revealed no significant three-way interaction between pre-natal exposure  $\times$  drug exposure  $\times$  treatment [ $F(1.36) = 0.8569$ ,  $p = 0.3608$ ], two-way interactions between pre-natal exposure  $\times$  drug exposure [ $F(1.36) = 2.157$ ,  $p = 0.1506$ ], drug exposure  $\times$  treatment [ $F(1.36) = 0.6406$ ,  $p = 0.4287$ ], pre-natal exposure  $\times$  treatment [ $F(1.36) = 0.1686$ ,  $p = 0.6838$ ] or significant effects of pre-natal exposure [ $F(1.36) = 0.01400$ ,  $p = 0.9065$ ], drug exposure [ $F(1.36) = 0.9630$ ,  $p = 0.3330$ ] and treatment [ $F(1.36) = 0.008186$ ,  $p = 0.9284$ ]. Tukey's multiple comparisons post-hoc analysis indicated no significant differences between the groups (Figure 7A). A large practical trend towards a decrease in striatal PPP1R1B was observed in the LPS-EFV-SAL (Cohen's  $d$ -value = 0.83) group compared to the control (SAL-OO-SAL) group (Figure 7A).

Frontal cortical PPP1R1B: Three-way ANOVA revealed a significant two-way interaction between pre-natal exposure  $\times$  drug exposure [ $F(1.40) = 4.518$ ,  $p = 0.0398$ ]. No significant three-way

interaction between pre-natal exposure x drug exposure x treatment [ $F(1,40) = 0.01272$ ,  $p = 0.9108$ ], two-way interactions drug exposure x treatment [ $F(1,40) = 0.4463$ ,  $p = 0.5080$ ], pre-natal exposure x treatment [ $F(1,40) = 0.2987$ ,  $p = 0.5877$ ] or effects of pre-natal exposure [ $F(1,40) = 0.01694$ ,  $p = 0.8971$ ], drug exposure [ $F(1,40) = 0.02717$ ,  $p = 0.8699$ ] and treatment [ $F(1,40) = 1.808$ ,  $p = 0.1863$ ]. Tukey's multiple comparisons post-hoc analysis indicated no significant differences between the groups (Figure 7B).

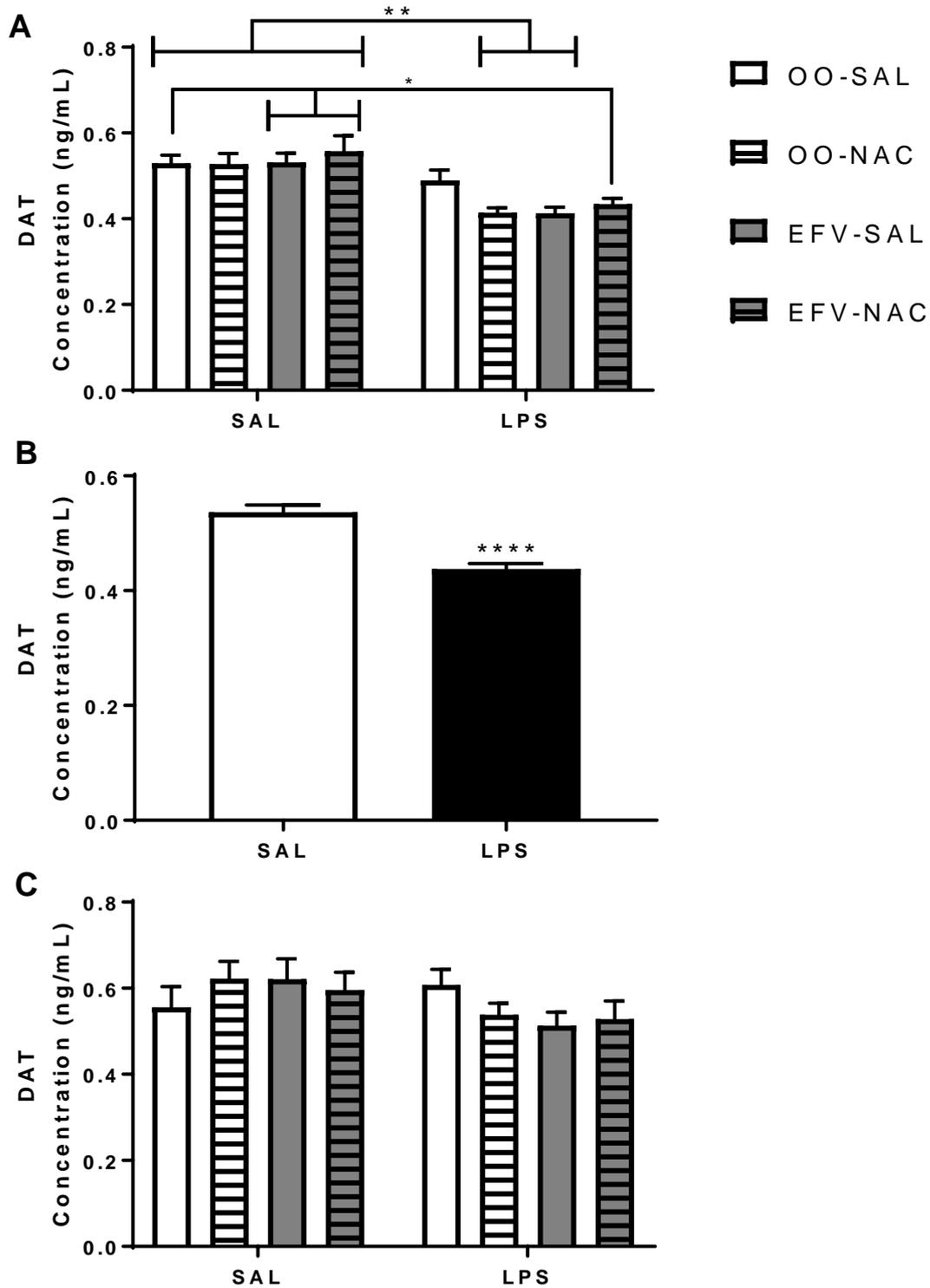


**Figure 7 A-B.** Phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B): **A** Striatal PPP1R1B: PPP1R1B concentration levels in the striatum of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test). # $d > 0.8$ , vs. the saline-olive oil-saline (SAL-OO-SAL) group (Cohen's  $d$ -value). **B** Frontal cortical PPP1R1B: PPP1R1B concentration levels in the frontal cortex of rats after exposure pre-natal LPS exposure and/or post-natal EFV exposure as well as NAC treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test).

**DAT**

Striatal DAT: Three-way ANOVA revealed a significant two-way interaction between drug exposure x treatment [ $F(1.88) = 4.139$ ,  $p = 0.0449$ ] and a significant effect of pre-natal exposure [ $F(1.88) = 41.33$ ,  $p < 0.0001$ ]. No significant three-way interaction between pre-natal exposure x drug exposure x treatment [ $F(1.88) = 1.237$ ,  $p = 0.2691$ ], two-way interactions between pre-natal exposure x drug exposure [ $F(1.88) = 2.067$ ,  $p = 0.1541$ ], pre-natal exposure x treatment [ $F(1.88) = 1.626$ ,  $p = 0.2057$ ] or the effects of drug exposure [ $F(1.88) = 0.1656$ ,  $p = 0.6851$ ] and treatment [ $F(1.88) = 0.2657$ ,  $p = 0.6076$ ], were observed. Tukey's multiple comparisons post-hoc analysis indicated a significant decrease in striatal DAT concentration in the LPS-OO-NAC group compared to SAL-OO-SAL ( $p = 0.0069$ ), SAL-OO-NAC ( $p = 0.0089$ ), SAL-EFV-SAL ( $p = 0.0057$ ) and SAL-EFV-NAC ( $p = 0.0003$ ) (Figure 8A). A significant decrease in in striatal DAT concentration in the LPS-EFV-SAL group compared to SAL-OO-SAL ( $p = 0.0063$ ), SAL-OO-NAC ( $p = 0.0082$ ), SAL-EFV-SAL ( $p = 0.0052$ ) and SAL-EFV-NAC ( $p = 0.0003$ ) (Figure 8A). Also, a significant decrease in striatal DAT concentration in the LPS-EFV-NAC group compared to SAL-OO-SAL ( $p = 0.0481$ ), SAL-EFV-SAL ( $p = 0.0415$ ) and SAL-EFV-NAC ( $p = 0.0030$ ) (Figure 8A). Unpaired student's t-test revealed a significant decrease in striatal DAT concentrations between the animals exposed to pre-natal LPS and the SAL group ( $p < 0.0001$ ) (Figure 8B).

Frontal cortical DAT: Three-way ANOVA revealed no significant three-way interaction between pre-natal exposure x drug exposure x treatment [ $F(1.88) = 2.455$ ,  $p = 0.1207$ ], two-way interactions between pre-natal exposure x drug exposure [ $F(1.88) = 1.612$ ,  $p = 0.2075$ ], drug exposure x treatment [ $F(1.88) = 0.004467$ ,  $p = 0.9469$ ], pre-natal exposure x treatment [ $F(1.88) = 0.7293$ ,  $p = 0.3954$ ] or significant effects of pre-natal exposure [ $F(1.88) = 3.419$ ,  $p = 0.0678$ ], drug exposure [ $F(1.88) = 0.3442$ ,  $p = 0.5589$ ] and treatment [ $F(1.88) = 0.01241$ ,  $p = 0.9116$ ]. Tukey's multiple comparisons post-hoc analysis indicated no significant differences between the groups (Figure 8C).



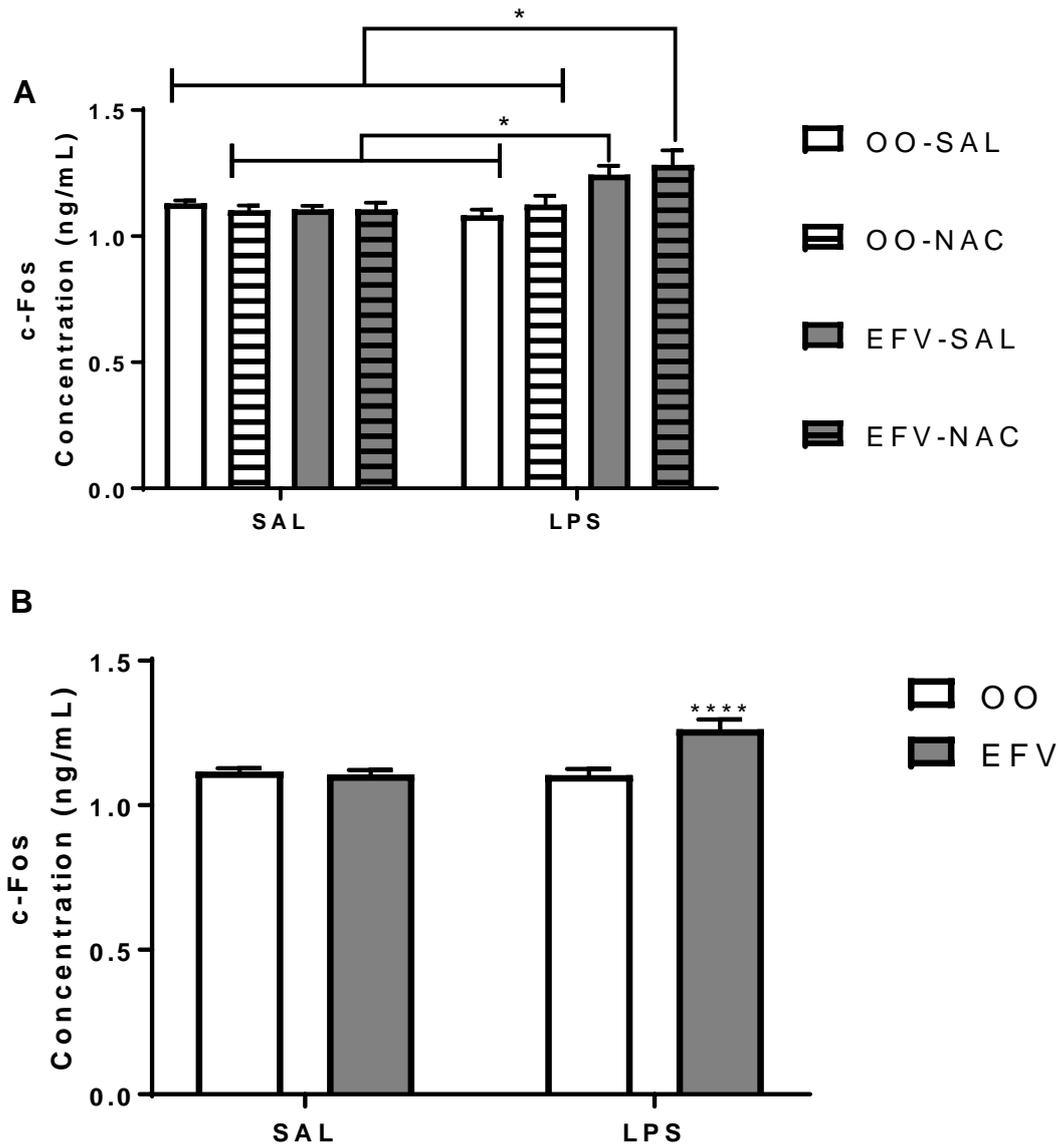
**Figure 8 A-C Dopamine transporters: A** Striatal DAT: DAT concentration levels in the striatum of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \*\* $p < 0.01$  LPS-olive oil (OO)-NAC & LPS-EFV-saline (SAL) vs. all SAL groups, \* $p < 0.05$  LPS-EFV-NAC vs. SAL-OO-SAL, SAL-EFV-SAL, SAL-EFV-NAC (three-way ANOVA, Tukey's multiple comparisons test). **B** Striatal DAT: DAT concentration levels in the striatum of rats after pre-natal exposure to LPS or SAL. Data

are expressed as mean  $\pm$  SEM of 48 animals per group. \*\*\*\* $p < 0.0001$  vs. SAL (Unpaired student's t-test).

**C Frontal cortical DAT:** DAT concentration levels in the frontal cortex of rats after exposure pre-natal LPS exposure and/or post-natal EFV exposure as well as NAC treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group (three-way ANOVA, Tukey's multiple comparisons test).

### **c-Fos**

Three-way ANOVA revealed a significant two-way interaction between pre-natal exposure x drug exposure [F(1.88) = 15.02,  $p = 0.0002$ ] and significant effects of pre-natal exposure [F(1.88) = 11.03,  $p = 0.0013$ ] and drug exposure [F(1.88) = 11.68,  $p = 0.0010$ ]. No significant three-way interaction between pre-natal exposure x drug exposure x treatment [F(1.88) = 0.1181,  $p = 0.7319$ ], two-way interactions between drug exposure x treatment [F(1.88) = 0.07143,  $p = 0.7899$ ], pre-natal exposure x treatment [F(1.88) = 1.54,  $p = 0.2180$ ] or an effect of treatment [F(1.88) = 0.3969,  $p = 0.5303$ ], were observed. Tukey's multiple comparisons post-hoc analysis indicated a significant increase in the c-Fos concentration in the LPS-EFV-NAC group compared to the SAL-OO-SAL ( $p = 0.0155$ ), SAL-OO-NAC ( $p = 0.0021$ ), SAL-EFV-SAL ( $p = 0.0027$ ), SAL-EFV-NAC ( $p = 0.0027$ ), LPS-OO-SAL ( $p = 0.0004$ ) and LPS-OO-NAC ( $p = 0.0115$ ) groups (Figure 9A). A significant increase in the c-Fos concentration in the LPS-EFV-SAL group compared to the SAL-OO-NAC ( $p = 0.0358$ ), SAL-EFV-SAL ( $p = 0.0443$ ), SAL-EFV-NAC ( $p = 0.0443$ ) and LPS-OO-SAL ( $p = 0.009$ ) groups (Figure 9A). Two-way ANOVA revealed a significant two-way interaction between pre-natal exposure x drug exposure [F(1.92) = 15.33,  $p = 0.0002$ ] and significant effects of pre-natal exposure [F(1.92) = 11.26,  $p = 0.0012$ ] and drug exposure [F(1.92) = 11.92,  $p = 0.0008$ ]. Tukey's multiple comparisons post-hoc analysis indicated a significant increase in the c-Fos concentration in the LPS-EFV group compared to SAL-OO ( $p < 0.0001$ ), SAL-EFV ( $p < 0.0001$ ) and LPS-OO ( $p < 0.0001$ ) (Figure 9B).



**Figure 9 A-B.** Cerebellar c-Fos **A:** Cerebellar c-Fos: c-Fos concentration levels in the cerebellum of rats after exposure pre-natal lipopolysaccharide (LPS) exposure and/or post-natal efavirenz (EFV) exposure as well as N-acetylcysteine (NAC) treatment. Data are expressed as mean  $\pm$  SEM of 12 animals per group. \*\* $p < 0.01$  vs. LPS-EFV-saline (SAL) & LPS-EFV-NAC, \* $p < 0.05$  vs. LPS-EFV-NAC & SAL groups (three-way ANOVA, Tukey's multiple comparisons test). **B:** Cerebellar c-Fos: c-Fos concentration levels in the cerebellum of rats after exposure pre-natal LPS exposure and/or post-natal EFV exposure. Data are expressed as mean  $\pm$  SEM of 24 animals per group. \*\*\*\* $p < 0.0001$  vs. SAL-olive oil (OO), SAL-EFV and LPS-OO (three-way ANOVA, Tukey's multiple comparisons test).

#### 4. Discussion

The main findings of this study include the observation that sub-acute EFV (5 mg/kg) exposure does not induce any addictive- or psychotic-like behaviour in rats. The peripheral (GSH & CORT) and neurochemical (DARPP-32 (PPP1R1B) & DAT) data were in accordance with the observed behaviour and remained unchanged in the EFV exposure group. Pre-natal LPS induced hyper-locomotion and aversive behaviour in the CPP, as well as deficits in %PPI. Pre-natal LPS decreased striatal DAT. Pre-natal LPS exposure in combination with post-natal EFV (LPS+EFV) exposure induced hyper-locomotion in the CPP, although not more significant than LPS exposure alone. LPS+EFV also induced %PPI deficits, although not more significant than either exposure alone. Peripherally, CORT was increased in LPS+EFV, NAC alone and LPS+EFV+NAC groups while GSH decreased in the groups receiving post-natal EFV exposure and NAC treatment irrespective of the pre-natal exposure (LPS or SAL). LPS+EFV increased cerebellar c-Fos and decreased striatal DAT and striatal DARPP-32(PPP1R1B). Frontal cortical DAT and DARPP-32 (PPP1R1B) were unaffected in all groups. NAC treatment did not reverse any of the effects induced by LPS, EFV and LPS+EFV exposure.

The CPP is a behavioural test that investigates reinforcing or aversive effects brought on by pharmacological substances (Lammel et al., 2012) and has been used by numerous researchers investigating addiction (Marusich et al., 2017, Nelson et al., 2017, Serna et al., 2019, Markos et al., 2018). Pre-natal LPS exposure significantly decreased the time spent in the drug-paired compartment(s) compared to pre-natal SAL exposure, thus inducing conditioned place aversion. This could possibly be attributable to LPS inducing hyper-locomotor activity, a behavioural alteration evident in neurodevelopmental models of SCZ (Basta-Kaim et al., 2011a, Howland et al., 2012) and regarded as a positive SCZ symptom (Canever et al., 2010, Simões et al., 2018), such as psychotic agitation (Powell et al., 2009). Said hyperactivity increases the likelihood of the animal exiting the drug-paired compartment in the testing phase, so that any possible reinforcing processes may be masked (Huston et al., 2013b). Another argument for the LPS induced conditioned place aversion might be that CPP is a test of learning and reference memory indicating cognitive, motivated preference behaviour (Galea et al., 2001), while pre-natal LPS exposure is known to induce numerous cognitive and learning memory deficits (Wischhof et al., 2015b, Hao et al., 2010).

Turning to sub-acute low dose 5 mg/kg EFV exposure, it did not produce any rewarding properties in the CPP. This is in line with previous work (Gatch et al., 2013) although differs from that of others (Möller et al., 2018). However, these results correlates with the behavioural profiles of hallucinogens, LSD (Parker, 1996) and 5-methoxy-a-methyltryptamine (Abiero et al., 2019), which also failed to produce a rewarding effect in the CPP at low dosages. Sub-acute exposure to EFV also did not alter locomotor activity, which correlates with several pre-clinical studies in rats

exposed to acute- (3 mg/kg (Gatch et al., 2013)) and sub-chronic EFV (5 mg/kg (Möller et al., 2018) & 10 mg/kg (Romao et al., 2011)). Since hallucinogenic drugs fail to present with dependence properties or produce addiction (Nichols, 2004), EFV (sub-acute exposure at a dose of 5 mg/kg) may present more as a hallucinogenic and not a psychostimulant. The combination of LPS+EFV were not successful in producing rewarding properties within the CPP. Thus, exposure to sub-acute EFV in combination with a contributory factor (LPS), indicated no addictive-like behaviour despite previous literature indicating the ability of MIA to potentiate the vulnerability towards addiction (Borçoi et al., 2015).

Deficits in %PPI is generally acknowledged as an endophenotype of SCZ (Braff and Light, 2005), being considered the “interface of cognition and psychosis” (Desbonnet et al., 2009) where deficits in sensory motor gating (cognitive impairment) may contribute towards psychotic symptoms (Togay et al., 2019, Parwani et al., 2000). In this study pre-natal LPS exposure induced significant %PPI deficits, in line with previous studies (Swanepoel et al., 2018, Wischhof et al., 2015b). However sub-acute EFV exposure did not induce any %PPI deficits. These data are congruent with another recent study that evaluated the effects of perinatal EFV (100 mg/kg) exposure on behavioural alterations later in life (Van de Wijer et al., 2019). Taken together, these findings indicate that EFV exposure, at the dosages tested, is not psychotogenic. Pre-natal LPS plus post-natal EFV exposure had a significant abrogating effect on %PPI. This coincide with a statement made by Meyer and colleagues which elaborated on the fact that MIA enhance sensitivity to hallucinogens as well as N-methyl-D-aspartate glutamine receptor antagonists (Meyer, 2013).

Interestingly, when considering the outcomes of the PPI (before treatment)- and locomotor activity (before and after treatment) test, all effects induced by LPS trended towards a stronger response by the addition of EFV. EFV in combination with pre-natal LPS exposure significantly increased the total distance moved and significantly decreased the %PPI when compared to the control group. EFV has been classified as a partial agonist at 5-HT<sub>2A</sub> receptors (Gatch et al., 2013). When given alone no significant effects were observed. However, when given to rats prenatally exposed to LPS, the LPS+EFV effects tended towards a stronger response. This may be attributed to LPS increasing the expression and sensitivity of 5-HT<sub>2A</sub> receptors within the pre-FC (PFC), as found by previous pre-clinical studies investigating pre-natal stress (Holloway et al., 2013) and pre-natal inflammation (Moreno et al., 2011, Wischhof et al., 2015a). A previous post-mortem study also found an upregulation of 5-HT<sub>2A</sub> in the PFC of untreated SCZ patients (Muguruza et al., 2013). Such an action may predispose patients to psychosis (González-Maeso et al., 2008). The activation of 5-HT<sub>2A</sub> (which is essential for psychoactive effects (González-Maeso et al., 2007)) will subsequently lead to an excessive glutamate (GLU) transmission within the PFC (Ham et al., 2017), resulting in an enhanced DA release in the NAcc (Murase et al., 1993) (within the ventral striatum (Wand et al., 2007)) and stimulation of D<sub>2</sub> receptors (Walter et al., 2009). This latter

mechanism can also be linked to the hyper-locomotion (Pijnenburg and Van Rossum, 1973) and decreased %PPI (Swerdlow et al., 1992, Powell et al., 2003) observed in the LPS+EFV groups, and emphasizes an increase in D<sub>2</sub> receptor signalling in these brain regions.

Considering the role of DARPP-32 (or PPP1R1B) in the phosphorylation activity of both D<sub>1</sub> and D<sub>2</sub> receptors within the FC and striatum (Wang et al., 2017), assessing this marker will ratify the above-mentioned DA-ergic hyperactivity. However, cortico-striatal PPP1R1B data showed no significant differences between groups except a large effect size ( $d = 0.83$ ) of a decrease in the striatum in the LPS+EFV group compared to control, possibly indicating that the combination of LPS and EFV induced dephosphorylation of DARPP-32(PPP1R1B) and hence decreased its concentration. This outcome is in line with our earlier proposal that the observed hyper-locomotion (Pijnenburg and Van Rossum, 1973) and decreased %PPI (Swerdlow et al., 1992, Powell et al., 2003) may be due to 5-HT<sub>2A</sub>-mediated D<sub>2</sub> stimulation in a pre-natal LPS plus post-natal EFV exposure model. The frontal cortical PPP1R1B and DAT data, showed no significant differences across any groups, indicating no hypo- or hyper DA-ergic activity within this brain area. With regards to SCZ this is interesting as it is a known fact that SCZ patients present with hypoactive mesocortical DA-ergic activity (Walter et al., 2009). However, available literature regarding the effects of pre-natal LPS on frontal cortical DA-ergic activity appears to be inconsistent. Previous pre-clinical studies utilising LPS reported either an increase (Swanepoel et al., 2018), decrease (Basta-Kaim et al., 2011b, Wang et al., 2009) or no change (Kirsten and Bernardi, 2017) in frontal cortical DA. This phenomenon cannot be attributed to either LPS dosages or time of pre-natal exposure and therefore demands further investigation.

LPS had a significant effect on striatal DAT levels and almost all groups prenatally exposed to LPS presented with lower striatal DAT levels when compared to most of the SAL groups. Pre-clinical research regarding the effects of pre-natal inflammation on DAT is insufficient and the available studies do not coincide with our findings: pre-natal LPS (100 µg/kg GD15/16) exposure did not alter DAT in the NAcc and striatum on PND60 (Baharnoori et al., 2013), and pre-natal polyinosinic-polycytidylic acid (5 mg/kg GD9) exposure as immunogen also found no alterations in DAT (NAcc) on PND 70 (Vuillermot et al., 2010). Clinical findings regarding DAT densities in SCZ patients irrespective of treatment are inconsistent, with a post-mortem study reporting a decrease in striatal DAT densities (Dean and Hussain, 2001) whereas imaging studies reported an increase (Artiges et al., 2017) as well as no differences in striatal DAT densities when comparing SCZ patients to healthy controls (Laakso et al., 2000, Fusar-Poli and Meyer-Lindenberg, 2012, Lavalaye et al., 2001). These inconsistencies warrant further investigation. That said, DA availability in the brain is mediated by DAT (Vaughan and Foster, 2013) as it plays an essential role in limiting DA transmission by clearing released DA back into pre-synaptic neurons (Leviel, 2011). When DAT is decreased, the clearance of DA is reduced (Best et al., 2009) resulting in an increased DA concentration within the extracellular space. The reduced

striatal DAT levels, observed in the majority of the LPS groups, correlates with the hyperdopaminergia hypothesis of SCZ where SCZ patients present with an increase in DA activity within the mesolimbic region resulting in positive SCZ symptoms (Walter et al., 2009, Gründer and Cumming, 2016). Furthermore, previous literature has reported a reduction in DAT function concurrent with mania-like behaviours (Milienne-Petiot et al., 2017). Therefore, we propose that the reduced striatal DAT levels induced by pre-natal LPS might contribute towards the presentation of hyper-locomotor activity and %PPI deficits. Sub-acute EFV exposure alone, however, did not induce any DAT alterations within the striatum. To the best of our knowledge, no pre-clinical studies have investigated the effects of EFV on DAT levels. Nevertheless, the effects observed of EFV on DAT further support the behavioural data in this study, viz. that EFV does not have reinforcing properties, nor does it present evidence for inducing psychosis-related behaviour. LPS+EFV presented with a decrease in striatal DAT. This outcome also concurred with the mania-like behaviour (locomotor activity (before and after treatment) and reduced %PPI (before treatment)) observed in the respective groups.

GSH serves as an excellent bio-marker for oxidative stress (Fung and Hardan, 2019). GSH is an endogenous antioxidant that scavenges ROS and RNS in order to restore redox homeostasis (Dean et al., 2011). Therefore, since GSH (or antioxidant capacity) depletion leads to a heightened ROS activity as well as oxidative stress (Vaziri et al., 2000), it can be said that the levels of GSH are inversely correlated with oxidative stress (or ROS/RNS levels). In this study pre-natal LPS exposure had no significant effects on GSH, which was surprising considering previous studies have indicated a decrease of GSH levels in SCZ patients (Raffa et al., 2011, Raffa et al., 2009). However, we could not find pre-clinical studies that investigated the effects of LPS on GSH in rat plasma, only studies investigating the effects of pre-natal LPS exposure on GSH levels in the brain. The latter studies all found a reduction in regional brain GSH levels (Al-Amin et al., 2016, Zhu et al., 2007, Ebaid et al., 2012, Lanté et al., 2008). Nevertheless, all the above-mentioned studies used higher LPS dosages (300 µg/kg, 10 000 endotoxin units/kg, 2.5 mg/kg & 500 µg/kg, respectively) as compared to a dosage of 100 µg/kg used in the current study, possibly indicating a lesser degree of MIA induced by LPS in our study. EFV exposure alone had no effect on GSH levels which is in accordance with a previous cell-culture study that observed no alterations in cellular- or extracellular GSH levels after EFV exposure (Brandmann et al., 2012). The combination of LPS and EFV also had no significant impact on GSH levels, although not surprising considering the impact of each exposure alone on GSH.

A recent pre-clinical study reported that physiological levels of ROS are of utmost importance to maintain homeostasis of the hypothalamic-pituitary-adrenal (HPA)-axis (Prevatto et al., 2017). Therefore, should a redox disturbance occur in any direction, it will result in the hyperactivity of the HPA-axis (Prevatto et al., 2017). Interestingly, a review article further reported on high glucocorticoid levels that can decrease antioxidant enzymes and in this manner maintain

oxidative stress (Schiavone et al., 2013). Although the response to NAC is discussed later, it is pertinent to note here a recent study that describes a hyperactive HPA-axis following prolonged exposure to an antioxidant, viz. NAC (150 mg/kg) (Prevatto et al., 2017). The authors attribute this to downregulating glucocorticoid receptors on the pituitary and upregulating the adrenocorticotrophic hormone receptor on the adrenal glands which subsequently resulted in an increased plasma level of CORT (Prevatto et al., 2017). The point here is that physiological manipulation of ROS, be it by chemical or other means (e.g. stress), is important for HPA-axis homeostasis, with a disturbance in ROS resulting in a hyperactive HPA-axis (Prevatto et al., 2017).

In this study pre-natal LPS exposure had a significant impact on plasma CORT levels as it greatly contributed towards the increase thereof. This outcome is in line with pre-clinical (Basta-Kaim et al., 2011a) and clinical findings (Yilmaz et al., 2007). Clearly, MIA and associated cytokine production affects the HPA-axis to increase CORT levels (Tilders et al., 1994). In this study, no changes to plasma CORT were observed after exposure to sub-acute EFV alone. No other pre-clinical investigation has considered the effects of EFV on CORT plasma levels. Nevertheless, one study found that chronic EFV (10 mg/kg) induced the release of pro-inflammatory cytokines (TNF- $\alpha$  & IL- $\beta$ ) (O'Mahony et al., 2005) that might in turn trigger an HPA-axis response (Dunn, 2000) and so increase the release of CORT. However, the latter study investigated the effects of chronic EFV (10 mg/kg) in healthy rats. Therefore, it should be considered that the level of cytokines produced as well as any subsequent HPA-axis response may be dependent on the type and/or dose of the immune adjuvant as well as exposure time. We observed an increase in plasma CORT levels in rats exposed to both LPS and EFV (a large effect size ( $d = 0.88$ )) versus control (SAL-OO-SAL). However, combined EFV+LPS did not show augmentative actions versus either exposure alone.

The link between redox and psychosis and/or addictive behaviour can also be appreciated if we consider the direct link to brain DA. An earlier study from our laboratory has described an elevation in striatal DA in a neurodevelopmental model of SCZ (post-weaning social isolation) (Möller et al., 2013b). Importantly though, the associated regional brain monoamine changes could be reversed by introducing an antioxidant as treatment, viz. NAC (150 & 250 mg/kg) (Möller et al., 2013b). The exact mechanism whereby cortisol/CORT may increase DA remains elusive but previous work demonstrates a variety of possibilities such as: a). reduced DA metabolism (Lindley et al., 1999), b). modifying post-synaptic DA transmission in the striatum (Barrot et al., 2001), c). some DA neurons possess corticosteroid receptors (Härfstrand et al., 1986), therefore the stimulation will also induce DA release, and d) CORT reduces DA uptake into synaptosomes (Gilad et al., 1987).

Finally, the presence of immediate early genes has been linked to brain DA function and to behavioural symptoms related to SCZ (Turgeon et al., 2007) and addiction (Carbo-Gas et al., 2014). To consider this, c-Fos concentration was evaluated as an indication of neuronal activity (Gallo et al., 2018) in the cerebellum. Similar to the addictive- and psychotic-like behaviour that became more apparent in the LPS+EFV groups, increased cerebellum c-Fos concentration was only observed when EFV was combined with pre-natal LPS exposure. Indeed c-Fos expression is facilitated by 5-HT<sub>2A</sub> activation which subsequently mediates the release of GLU (Scruggs et al., 2000). Although somewhat speculative, this may support our hypothesis that LPS exposure possibly upregulated and increased the sensitivity of the 5-HT<sub>2A</sub> receptors, and which is also acted upon by EFV as previously explained. Since 5-HT<sub>2A</sub> receptors are increased in the white matter of the cerebellum of SCZ patients (Eastwood et al., 2001), it is possible that pre-natal LPS, as a model of SCZ, similarly increased 5-HT<sub>2A</sub> receptor expression in the cerebellum. However, to the best of our knowledge no pre-clinical studies have previously investigated the effects of pre-natal inflammation on 5-HT<sub>2A</sub> expression in the cerebellum (as observed in the PFC) and therefore this speculative argument requires further investigation. That said, whole cerebellum was used for analysis in this study, indicating that non-specific cerebellar neuronal activity may be responsible for the behavioural alterations described, in particular hyper-locomotor activity (Morton and Bastian, 2004) and %PPI deficits (Schmahmann and Caplan, 2006)). Furthermore, this only took place when EFV was combined with LPS, thereby suggesting the involvement of the cerebellum in both psychotic and sensory motor gating behaviours following this particular exposure.

Considering the contributory role of oxidative stress in SCZ, addiction and in the neurotoxic profile of EFV, we attempted to reverse the above-mentioned bio-behavioural responses to pre-natal LPS exposure, post-natal EFV exposure as well as the combination thereof with the antioxidant, NAC.

The foundation of NAC's therapeutic efficacy is built on NAC's ability to regulate the biosynthesis of GSH (Berk et al., 2013). In this study, NAC treatment had a trend towards increasing GSH levels when compared to SAL treatment, despite any pre-natal challenges. Considering the study performed by Prevatto and colleagues (2017) (as discussed above), NAC might have decreased the physiological ROS/RNS levels within the animals which could explain the increased CORT level observed in the SAL-OO-NAC group. Interestingly, GSH plasma levels in rats exposed to sub-acute EFV and treated with chronic NAC were significantly lower when compared to rats only treated with chronic NAC. This may indicate that EFV can reduce or stop the ability of NAC to increase GSH levels and therefore the animals might present with higher ROS/RNS levels. However, the exact mechanism of this phenomenon remains elusive and warrants further investigation. The point is, NAC (100 mg/kg) had no therapeutic efficacy in this study and other mechanisms involved in the attenuation of %PPI deficits (induced by LPS alone and LPS+EFV)

hyper-locomotor activity (induced by LPS+EFV) and striatal PPP1R1B after NAC treatment should be considered.

Regarding the PPI data, no group presented with %PPI deficits after the NAC treatment regimen on PND 72. A possible explanation for these discrepancies between the PPI tests done before and after the NAC treatment regimen respectively might be that the first PPI tests were done on PND 55 and the second on PND 72: previous studies also found that pre-natal LPS had no significant effect on the PPI done on PND 72 (Simões et al., 2018, Dickerson and Bilkey, 2013, Gray et al., 2019). Another factor that must be taken into account is the possible familiarization with the PPI test, that might attribute to the unaltered PPI observed in the second part of the study. A similar outcome were previously found in a pre-clinical study (primates) evaluating a psychoactive substance, MK-801 (Saletti et al., 2015). In the study performed by Saletti and colleagues (2015) the animals underwent repeated PPI testing at a two-week test interval and despite showing a decrease in %PPI in the first test, it was not observed at the second PPI testing. Indeed, other studies focusing on learning and memory also reported on how familiarization of the testing environment can impact effects induced by psychoactive substances (Chan and McNally, 2009, Uekita and Okaichi, 2005, Shapiro and O'Connor, 1992).

Regarding the locomotor and striatal PPP1R1B data, the ability of NAC treatment to reverse the hyper-locomotor activity in the LPS+EFV group and PPP1R1B levels returning to normal may be attributed to the increased CORT plasma levels observed in the LPS-EFV-NAC group. The combination of LPS, EFV and NAC significantly increased plasma CORT compared to all the other groups, which may be attributed to a synergistic effect but also keeping in mind the EFV+NAC group presented with lower GSH levels that could've contributed towards the disturbance in the HPA-axis. Previous studies found that CORT has the ability to increase DA transmission within the striatum (Barrot et al., 2001, Lindley et al., 1999, Härfstrand et al., 1986, Gilad et al., 1987) whereas prolonged exposure to CORT may lead to an enhanced DA concentration within the striatum which subsequently results in a down-regulation of D<sub>2</sub> receptors (Lammers et al., 1999). Thus, the increased CORT in the LPS-EFV-NAC group may have increased the DA in such a manner that a down-regulation of DA receptors occurred as previous studies indicated a D<sub>2</sub> down-regulation after the repeated stimulation thereof (Chen et al., 1993). Therefore, despite having an increased amount of DA, the behavioural effects mediated by the D<sub>2</sub> (i.e. hyper-locomotion (Pijnenburg and Van Rossum, 1973)) will now be diminished. The PPP1R1B levels will however be normal in the LPS-EFV-NAC group as stimulation of the DA receptors will be to a lesser extent compared to the other groups. This is in line with the studies reported by Lindley and colleagues where glucocorticoids attenuated behaviours mediated by the D<sub>2</sub> (Lindley et al., 1999).

Considering the outcomes of NAC treatment in this study, we do not disregard the capacity of NAC being a viable treatment for psychiatric disorders as demonstrated abundantly by several pre-clinical and clinical researchers (Möller et al., 2013a, Möller et al., 2013b, Grant et al., 2010, Slattery et al., 2015, Chen et al., 2016). We do, however, suggest that the outcome observed in this study may be attributed to a dose-dependent effect of NAC. The NAC dosage (100 mg/kg) applied in the current study was based on a previous pre-clinical study investigating addiction (described in section 2.4) and not psychotic-like behaviour which evidently pose as a limitation in this study. Therefore, it is in our opinion that a higher NAC dose could've presented with a better outcome as Möller and colleagues observed a significant improvement in psychotic-like bio-behavioural alterations after utilizing NAC (150 and 250 mg/kg).

In conclusion, although sub-acute EFV exposure did not induce addiction or psychosis-related behaviours in healthy subjects, this study highlights the dangers of sub-acute EFV abuse in predisposed individuals. The latter would include individuals with a predisposition to developing a psychotic and/or addiction disorder later in life, e.g. exposure to early life adversity such as pre-natal infection.

### **List of abbreviations**

5-HT – Serotonin, ANOVA – Analysis of variance, ARRIVE - Animal Research: Reporting of In Vivo Experiments, ARV – Antiretroviral, CORT – Corticosterone, CPP – Conditioned place preference, DA – Dopamine, DARPP-32 - DA-and-cyclic adenosine monophosphate-regulated phosphoprotein (with a molecular weight of 32 kD), DAT – Dopamine transporters, EFV – Efavirenz, ELISA – Enzyme linked immunosorbent assay, FC – Frontal cortex, GD – Gestational day, GLU – Glutamate, GSH – Glutathione, HIV - Human immunodeficiency virus, HPA – Hypothalamic-pituitary-adrenal, LPS – Lipopolysaccharide, LSD – Lysergic acid diethylamide, MIA – Maternal immune activation, NAC – N-acetylcysteine, NAcc – Nucleus accumbens, NaCl – Sodium chloride, NRF - National Research Foundation, NWU – North-West University, OD – Optical density, OO – Olive oil, PBS - Phosphate buffered solution, PFC – Pre-frontal cortex, PND – Post-natal day, PPI – Pre-pulse inhibition, PPP1R1B - Phosphoprotein phosphatase-1 regulatory subunit 1B, RNS – Reactive nitrogen species, ROS – Reactive oxygen species, SAL – Saline, SC – Subcutaneously, SCZ – Schizophrenia, SD – Sprague-Dawley, SEM - standard error of the mean, SIPD – Substance-induced psychotic disorder.

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### **Author contributions**

Carmen Pieters (CP) undertook all behavioural and neurochemical studies, collected all data and undertook the statistical analysis. CP wrote the first draft of the manuscript. Marisa Möller (MM) supervised CP throughout the study and initiated and designed the study along with Brian H Harvey (BH). MM contributed towards the preparation of the final manuscript and BH provided critical revision of the manuscript. MM and BH approved the final manuscript for submission.

### **Conflict of interest**

The authors declare no conflict of interest regarding the publication of this article. We received EFV as a kind sponsorship from Aspen, South Africa. The study was funded and supported by the South African Medical Research Council (M. Möller) and the National Research Foundation (NRF) (NRF; M. Möller; Grant UID99276). The opinions, findings and conclusions or recommendations expressed in any publication generated by NRF supported research are those of the authors, and that the NRF accepts no liability whatsoever in this regard.

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## CHAPTER 4

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### CONCLUDING REMARKS, STUDY OUTCOMES, LIMITATIONS AND FUTURE RECOMMENDATIONS

#### 1.1 Concluding Remarks

*Efavirenz (EFV), a potential threat for developing psychosis and addiction?*

The advent of human immunodeficiency virus (HIV) / acquired immune deficiency syndrome (AIDS) 60 years ago (Melhuish and Lewthwaite, 2018) resulted in an epidemic which warranted a world-wide treatment intervention. Highly active antiretroviral treatment were set out in the 90's (Okwundu and Ogunjale, 2018) and HIV/AIDS became a manageable disease (Bezabhe et al., 2016). Nevertheless, a specific antiretroviral, EFV soon presented with adverse neuropsychiatric effects such as; anxiety, hallucinations, delusions and psychosis (Apostolova et al., 2017)). These effects hindered effective HIV/AIDS treatment as patients either discontinued their treatment regimen (Blanch et al., 2001) or, more disturbingly, abused the medication for the neuropsychiatric side-effects (Grelotti et al., 2014, Mokwena, 2015). This inspired the first part of the study, as it was necessary to investigate the consequences of EFV use especially with regards to a substance-induced psychotic disorder (which may ultimately lead to schizophrenia (SCZ) (O'Connell et al., 2019)) and/or EFV abuse/addiction. Previous pre-clinical studies reported on the psychedelic and addictive-like effects of EFV and attributed it to its distinct affinity for the serotonin (5-HT)<sub>2A</sub> receptor (Gatch et al., 2013) as well as its ability to increase cortico-striatal monoamines (Möller et al., 2018). Contributory to these mechanisms, EFV may promote oxidative stress (Adjene et al., 2010), by inducing pro-inflammatory cytokine production (O'Mahony et al., 2005) and subsequent cortisol release (Steensberg et al., 2003). Redox-inflammatory pathways (Upthegrove and Khandaker, 2019, Beiser and Yaka, 2019) as well as hypothalamic-pituitary-adrenal (HPA)-axis activity (Walker et al., 2008, Basta-Kaim et al., 2011, Wemm and Sinha, 2019) are altered in the respective disorders.

In this study addictive-like (as measured by the conditioned place preference (CPP) paradigm) and psychotic-like behaviour (as measured by locomotor activity and pre-pulse inhibition (PPI) tests) were not observed in the animals after sub-acute EFV (5 mg/kg) exposure (Figure 1A). Despite the surprising outcomes, it became evident how EFV correlates with the behavioural profile of hallucinogens (Figure 1A) (Parker, 1996, Abiero et al., 2019) and not psychostimulants. Importantly, the dose (5 mg/kg) of EFV was based on a previous study which also investigated addictive-like behaviour (Möller et al., 2018). Therefore, when considering the outcomes of the EFV with regards to psychotic-like behaviour as well as bio-markers measured, the dosage should be kept in mind (Figure 1A). Previous literature indicated the ability of EFV to induce

neuropsychiatric effects within patients (Arendt et al., 2007), however, clinical studies have shown a lower incidence of these effects when implementing either a reduced dose (Dickinson et al., 2015) or stepped-dosages (Gutierrez-Valencia et al., 2009). Sub-acute EFV exposure also had no significant impact on the peripheral- (corticosterone (CORT) and glutathione (GSH)) or neurochemical (c-Fos, dopamine transporters (DAT) and phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B)) bio-markers measured which concurred with the behaviour observed.

#### *Pre-natal inflammation, a contributory factor towards psychosis and addiction development?*

After an extensive literature review, several factors that contribute towards psychiatric disorders were noted, but especially fascinating was the influence of early life stress on the development of SCZ and addiction. A second part of this study emerged and focussed on pre-natal inflammation (as induced by lipopolysaccharide (LPS)) as a contributory factor (Wischhof et al., 2015, Straley et al., 2017). The pre-natal inflammation model also served as a neurodevelopmental model for SCZ (Estes and McAllister, 2016). Therefore, this study investigated pre-natal inflammation not only as a contributory factor towards the respective disorders, but it also investigated the effects of EFV in a model of SCZ. This ultimately enabled an investigation of a possible bi-directional association as well as an indicator as to how predisposed subjects would react to EFV use. Furthermore, as indicated in chapter 2, dysfunction of the frontal cortex (FC) and striatum is evident in SCZ (Liddle and Pantelis, 2003) and addiction (Weiss, 2005). However, this study explored outside the regions normally inflicted and chose to investigate the cerebellum and its involvement in the respective disorders by evaluating the neuronal activity (as indicated by c-Fos (Gallo et al., 2018)) in the cerebellum after exposure to all pre-natal factors (LPS vs Saline), pharmacological substances and behavioural testing.

As mentioned earlier, no addictive-like behaviour was observed in this study and therefore the discussion will continue with regards to psychotic-like behaviour. In this study, the pre-natal LPS (100 µg/kg) exposure induced SCZ-like behaviour (i.e. hyper-locomotor activity (on post-natal day (PND) 55 and 72) and average %PPI deficits (on PND55, but not on PND 72) and contributed significantly towards the decrease of striatal DAT (Figure 1B) and increase of plasma CORT. For a comprehensive discussion on the relations between bio-behavioural alterations and SCZ see chapter 3. Surprisingly, LPS induced no plasma GSH concentration alterations which might indicate that maternal immune activation occurred to a lesser extent as expected (see chapter 3 for explanation). Interestingly, in this study the bio-behavioural effects induced by LPS became more apparent after sub-acute EFV exposure (LPS+EFV). In the LPS+EFV group a decrease in %PPI (PND 55) along with increased locomotor activity (PND 55 and 72) were observed (Figure 1C). This outcome led to the suggestion of the 5-HT<sub>2A</sub> hypothesis explained in chapter 3, which is supported by the decrease in striatal PPP1R1B observed in the same group (Figure 1C). The

LPS+EFV group also presented with a significant increase in plasma CORT as well as decreased striatal DAT (Figure 1C). Interestingly, the LPS+EFV group was the only group to present with increased cerebellum c-Fos concentrations (Figure 1C), which led to the conclusion that the cerebellum does indeed play a role within psychotic-like behaviour as observed in the respective group. For a comprehensive explanation on c-Fos, see chapter 3.

#### *N-acetylcysteine (NAC), a potential treatment for psychosis and addiction?*

To the best of our knowledge, no potential treatment platform exists for these disorders induced by EFV (alone or in combination with pre-natal inflammation), and therefore a third part of this study was developed to investigate NAC (antioxidant and GSH precursor (Dean et al., 2011)) as a possible treatment option. NAC served as a viable option as it has the ability to influence all factors altered by EFV (Dean et al., 2011) and deemed promising after reviewing pre-clinical- and clinical studies using NAC as treatment in psychiatric disorders (Möller et al., 2013, Grant et al., 2010, Slattery et al., 2015, Chen et al., 2016).

In this study, average %PPI deficits as well as hyper-locomotor activity were attenuated after chronic NAC (100 mg/kg) treatment in the LPS+EFV group (Figure 1C). However, when EFV were combined with NAC treatment, the GSH levels were significantly decreased compared to NAC treatment alone (Figure 1C). This led to the conclusion that NAC is not responsible for the attenuated behaviour observed (Figure 1C). Furthermore, significant increases in plasma CORT were observed in the SAL-OO-NAC and LPS+EFV-NAC groups (Figure 1C). This indicated how chronic NAC treatment, and the combination of LPS, EFV and NAC impacted HPA-axis activity (for more information see chapter 3). As explained in chapter 3, increased CORT alter dopamine (DA) activity in the striatum (Barrot et al., 2001, Lindley et al., 1999) and it was therefore proposed that the high levels of CORT observed in the LPS+EFV-NAC group were responsible for the attenuated psychotic behaviour observed after NAC treatment (Figure 1C).

In this study, frontal cortical DAT and PPP1R1B were the only biomarkers that remained unaffected after pre-natal LPS exposure, post-natal EFV exposure and post-natal NAC treatment, which indicated no DA-ergic alterations within the respective area.

For a summary of all the findings in this study please see table 1 and for a possible mechanistic summary see the graphical representation, figure 1.

To conclude, this study ultimately indicated how existing neuropsychiatric effects became more apparent after sub-acute EFV exposure. This ultimately highlights the risks of EFV in predisposed individuals not only with regards to a possible psychotic disorder, but also to predisposed individuals in need of HIV/AIDS treatment. NAC was futile in this regard and further studies are

needed to establish a treatment platform that can diminish these effects induced by pre-natal adversity and EFV.

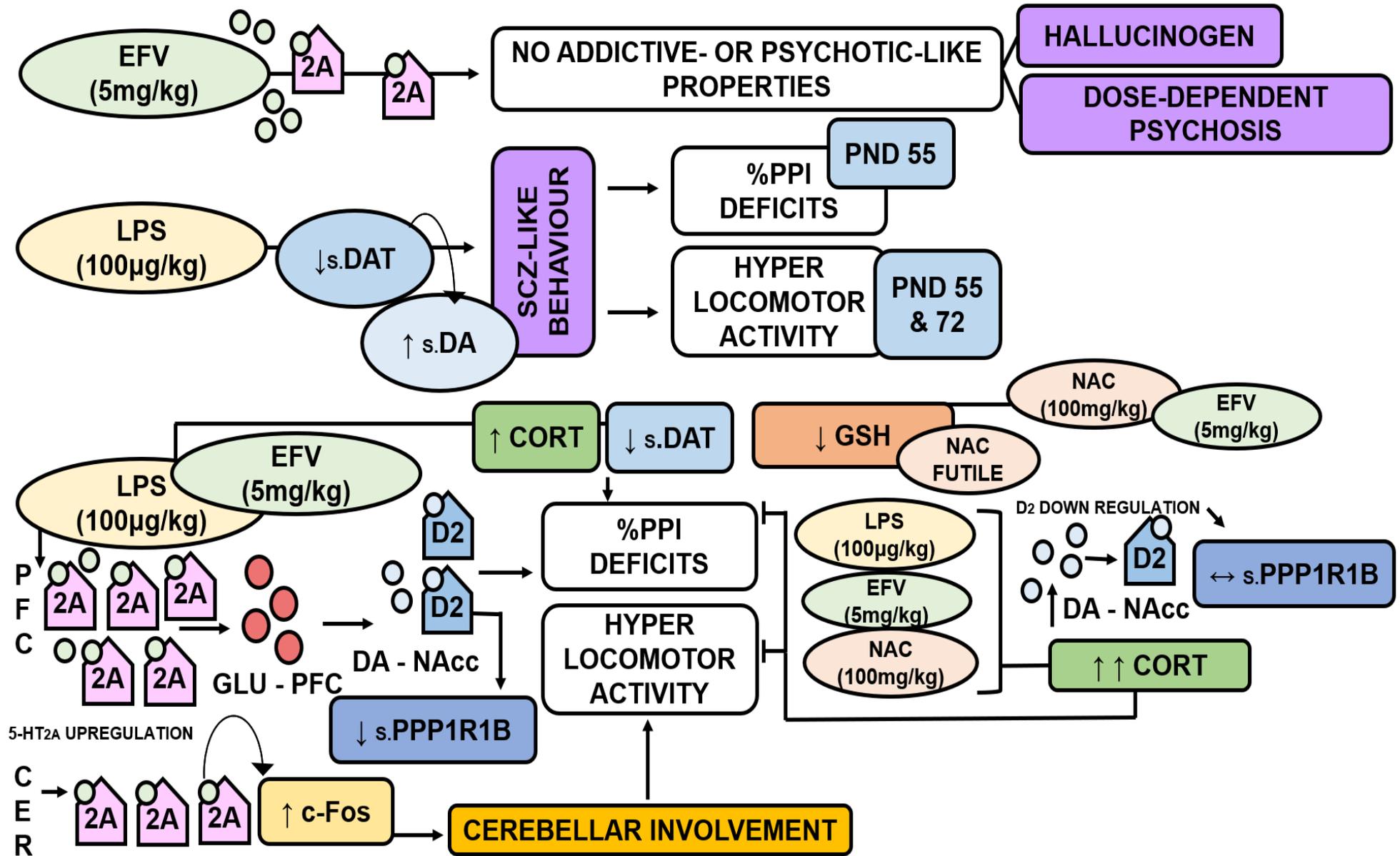
**Table 1:** A summary of bio-behavioural findings in this study.

BEFORE NAC TREATMENT						
BEHAVIOURAL OUTCOMES – AS DETERMINED VIA TWO-WAY ANOVA						
VS.		CPP (TIME SPENT IN DRUG-PAIRED COMPARTMENT (s))	LOCOMOTOR ACTIVITY (DISTANCE TRAVELED (cm))	PPI (%PPI)		
SAL-EFV	SAL - OO	↔	↔	↔		
LPS-OO		↔	↑↑	↔		
LPS-EFV		↔	↑↑↑	↓		
AS DETERMINED VIA T-TEST						
VS.		CPP (TIME SPENT IN DRUG-PAIRED COMPARTMENT (s))	LOCOMOTOR ACTIVITY (DISTANCE TRAVELED (cm))	PPI (%PPI)		
LPS	SAL	↓	↑↑↑↑	↓		
AFTER NAC TREATMENT						
BEHAVIOURAL OUTCOMES – AS DETERMINED VIA THREE-WAY ANOVA						
VS.		CPP (TIME SPENT IN DRUG-PAIRED COMPARTMENT (s))	PPI (%PPI)	VS.		LOCOMOTOR ACTIVITY (DISTANCE TRAVELED (cm))
SAL-OO-NAC	SAL - OO - SAL	↔	↔	LPS-OO-NAC	SAL-OO-NAC	↑↑
SAL-EFV-SAL		↔	↔	LPS-EFV-SAL	SAL-OO-NAC	↑↑↑↑
SAL-EFV-NAC		↔	↔		SAL-EFV-SAL	↑
LPS-OO-SAL		↔	↔		SAL-EFV-NAC	↑
LPS-OO-NAC		↔	↔		LPS-EFV-NAC	↑
LPS-EFV-SAL		↓↓	↔			
LPS-EFV-NAC		↓	↔			
AS DETERMINED VIA TWO-WAY ANOVA						
VS.		CPP				

		(TIME SPENT IN DRUG-PAIRED COMPARTMENT (s))		
SAL-NAC	SAL - SAL	↔		
LPS-SAL		⇓		
LPS-NAC		↓		
AFTER NAC TREATMENT				
PERIPHERAL OUTCOMES – AS DETERMINED VIA THREE-WAY ANOVA				
vs.		CORT (CONCENTRATION ng/ml)	GSH (CONCENTRATION µg/ml)	
SAL-OO-NAC	SAL - OO - SAL	↑	↔	
SAL-EFV-SAL		↔	↔	
SAL-EFV-NAC		↔	↔	
LPS-OO-SAL		↔	↔	
LPS-OO-NAC		↔	↔	
LPS-EFV-SAL		↑	↔	
LPS-EFV-NAC		↑	↔	
PERIPHERAL OUTCOMES – AS DETERMINED VIA TWO-WAY ANOVA				
vs.		GSH (CONCENTRATION µg/ml)		
EFV-NAC	OO-NAC	↓		
NEUROCHEMICAL OUTCOMES – AS DETERMINED VIA THREE-WAY ANOVA				
vs.		DAT (FC) (CONCENTRATION ng/ml)	PPP1R1B (FC) (CONCENTRATION ng/ml)	PPP1R1B (STRIATUM) (CONCENTRATION ng/ml)
SAL-OO-NAC	SAL - OO - SAL	↔	↔	↔
SAL-EFV-SAL		↔	↔	↔
SAL-EFV-NAC		↔	↔	↔
LPS-OO-SAL		↔	↔	↔
LPS-OO-NAC		↔	↔	↔
LPS-EFV-SAL		↔	↔	↓
LPS-EFV-NAC		↔	↔	↔

vs.		DAT (STRIATUM) (CONCENTRATION ng/ml)	vs.	c-FOS (CEREBELLUM) (CONCENTRATION ng/ml)	
LPS-OO-NAC & LPS-EFV-SAL	SAL-OO-SAL	↓↓	LPS - EFV - NAC	SAL-OO-SAL	↑
	SAL-OO-NAC	↓↓		SAL-OO-NAC	↑↑
	SAL-EFV-SAL	↓↓		SAL-EFV-SAL	↑↑
	SAL-EFV-NAC	↓↓		SAL-EFV-NAC	↑↑
LPS-EFV-NAC	SAL-OO-SAL	↓		LPS-OO-SAL	↑↑↑
	SAL-EFV-SAL	↓		LPS-OO-NAC	↑
	SAL-EFV-NAC	↓		LPS-EFV-SAL	↔
<b>NEUROCHEMICAL OUTCOMES – AS DETERMINED VIA TWO-WAY ANOVA</b>					
vs.		c-FOS (CEREBELLUM) (CONCENTRATION ng/ml)			
LPS-EFV	SAL-OO	↑↑↑			
	SAL-EFV	↑↑↑			
	LPS-OO	↑↑↑			

↑ -Significant increase, ↓ - Significant decrease, ↔ No alteration, x Not applicable. Abbreviations: NAC – N-acetylcysteine, ANOVA – Analysis of variance, SAL- Saline, EFV- Efavirenz, LPS – Lipopolysaccharide, CPP – Conditioned place preference, PPI – Pre-pulse inhibition, CORT – Corticosterone, GSH – Glutathione, DAT – Dopamine transporter, PPP1R1B - Phosphoprotein phosphatase-1 regulatory subunit 1B and FC – Frontal cortex.



**Figure 1:** A graphical representation of the main bio-behavioural effects observed in this study. A- Sub-acute exposure to efavirenz (EFV), a partial agonist of serotonin 2A receptors (2A or 5-HT<sub>2A</sub>), does not induce addictive- or psychotic-like bio-behavioural alterations. B- Pre-natal exposure to lipopolysaccharide (LPS) induced schizophrenia (SCZ)-like behaviour (hyper-locomotor activity (post-natal day (PND) 55 & 72) and %pre-pulse inhibition (PPI) deficits (PND 55)). One possible mechanism may be ascribed to LPS decreasing striatal dopamine transporters (s.DAT) which resulted in an increase in striatal dopamine (s.DA). C- LPS+EFV exposure: Effects (hyperlocomotor activity and %PPI deficits) induced by LPS became more apparent after EFV exposure (PND 55). This is possibly due to LPS inducing an upregulation of 2A and resulting in an increased response to EFV. LPS+EFV induced an increase in plasma corticosterone (CORT) and decreased s.DAT, all alterations which could contribute towards the SCZ-like behaviour. LPS+EFV also induced an increase in cerebellar c-Fos, which may also be attributed to the upregulation of 2A induced by LPS. This indicated cerebellar involvement in the %PPI deficits and hyper-locomotor activity observed. Behavioural (hyper-locomotor activity and %PPI deficits) and neurochemical (striatal phosphoprotein phosphatase-1 regulatory subunit 1B (s.PPP1R1B)) alterations were attenuated after the N-acetylcysteine (NAC) treatment regimen (PND 72). However, NAC proved to be futile in this study as glutathione (GSH) were reduced when NAC were combined with EFV. Other options were considered such as the effects of LPS+EFV+NAC on plasma CORT levels. The hyper-CORT resulted in an increased dopamine (DA) release within the striatum which could have resulted in the downregulation of the DA<sub>2</sub> receptor (D2). Abbreviations: PFC – Pre-frontal cortex, GLU- Glutamate, NAcc- Nucleus accumbens, CER- Cerebellum. Keys: green dots – EFV, red dots – GLU, blue dots – DA. Blocked lines indicate an attenuation of behaviour

## 1.2 Study outcomes

1. Objective: To investigate whether sub-acute EFV (5 mg/kg) exposure in rats will induce addictive-like behaviour, as found and validated by a previous study performed in our laboratory (Möller et al., 2018). Addictive-like behaviour will be determined using the CPP paradigm.

Outcome: The animals exposed to sub-acute EFV did not spend more time in the drug-paired compartment as compared to the control group. Therefore, EFV did not induce addictive-like behaviour.

2. Objective: To investigate possible psychotic-like behaviour in rats following sub-acute EFV (5 mg/kg) exposure. This behaviour will be assessed using the PPI test.

Outcome: Sub-acute EFV exposure did not induce a reduction in %PPI as compared to the control group. Therefore, EFV did not induce psychotic-like behaviour.

3. Objective: To investigate possible locomotor alterations induced by EFV (5 mg/kg). This test is measured in the CPP apparatus and can be an indicator to both addictive- and psychotic-like behaviour.

Outcome: Sub-acute exposure to EFV did not alter the distance travelled within the CPP apparatus as compared to the control group. This is a further indication of sub-acute EFV exposure not inducing psychotic effects as hyper-locomotion is connected to manic behaviour (Minassian et al., 2010).

4. Objective: To determine whether sub-acute EFV (5 mg/kg) exposure alone will induce alterations in peripheral corticosterone and GSH levels, cerebellar c-Fos, as well as alterations to DAT and PPP1R1B expression in the striatum and FC of rats.

Outcome: Animals exposed to sub-acute EFV induced no alterations within the peripheral- (CORT & GSH) or neurochemical (c-Fos, DAT & PPP1R1B) bio-markers measured.

5. Objective: To investigate whether an early life stressor (pre-natal inflammation induced by LPS (100 µg/kg)) alone will induce psychotic-like (determined using locomotor activity and the PPI test) behaviour in rats later in life, as previously found and validated in our laboratory (Swanepoel et al., 2018, Harvey et al., 2018).

Outcome: Pre-natal exposure to LPS had a main effect on the PPI and significantly reduced the %PPI as well as induced hyper-locomotion with an increase in distance travelled compared to the control group. Therefore, pre-natal exposure to LPS induced psychotic-like behaviour.

6. Objective: To investigate whether early life pre-natal inflammation (induced by LPS (100 µg/kg)) could contribute towards / exacerbate the above-mentioned bio-behavioural alterations (if any) in rats exposed to sub-acute EFV (5 mg/kg).

Outcome: In this study it was evident that effects induced by LPS became more apparent when the animals were also exposed to sub-acute EFV. These animals displayed an increase in distance travelled as well as a decrease in %PPI compared to the control group. An increase in plasma CORT and cerebellar c-Fos along with a decrease in striatal PPP1R1B and DAT were observed in groups exposed to LPS + EFV compared to the control group.

7. Objective: To establish whether chronic NAC (100 mg/kg) treatment can reverse sub-acute EFV induced bio-behavioural alterations in rats as mentioned above.

Outcome: Sub-acute EFV exposure did not induce any bio-behavioural alterations in this study. This outcome remained unchanged after the chronic NAC treatment.

8. Objective: To establish if chronic NAC (100 mg/kg) treatment could reverse bio-behavioural alterations induced by LPS (pre-natal inflammation) alone in rats.

Outcome: LPS induced psychotic-like behaviour (hyper-locomotor activity and PPI deficits) in the animals along with significantly affecting plasma CORT and striatal DAT. PPI deficits were attenuated after NAC treatment, but the hyper-locomotor activity persisted as pre-natal LPS exposure still had a significant impact on the distance travelled. NAC treatment did not restore the LPS induced CORT or DAT alterations.

9. Objective: To determine if chronic NAC (100 mg/kg) treatment could reverse bio-behavioural alterations induced by sub-acute EFV (5 mg/kg) in combination with pre-natal inflammation (induced by LPS) in rats.

Outcome: After NAC treatment the psychotic-like behaviour (PPI and locomotor activity) and striatal PPP1R1B levels concurred with those of the control group. Importantly, when EFV were combined with NAC, the GSH levels were significantly decreased when compared to NAC treatment alone. Therefore, NAC was possibly not responsible for the attenuated behaviours

induced by LPS+EFV. NAC did however, induce a further increase in plasma CORT, NAC treatment had no effect on cerebellar c-Fos or striatal DAT.

### **1.3 Limitations and future recommendations**

While this study addressed all the objectives presented in Chapter 1, it still presented with some limitations which should be considered for future studies. These limitations and future recommendations are discussed below;

1. In this study the CPP paradigm was used to evaluate the addictive-like behaviour as induced by sub-acute EFV exposure. This method was chosen after extensive research indicating its value, as well as the method being validated in our laboratory (Möller et al., 2018). Despite having faith in the CPP as a valuable method, I do believe it is a method that can be refined within our laboratory to produce more reliable results. I, therefore, firstly suggest a study which can refine the CPP in our laboratory. One possible recommendation could be to alter the physical structure of the apparatus. Secondly, I do believe that we should consider validating an alternative behaviour test (e.g. drug self-administration) in our laboratory suitable for investigating addictive-like behaviour. This would enable us to compare results from both tests and ultimately get a better picture of the addictive-like behaviour.
2. This study was based on the findings of Möller and colleagues in 2018. These findings had an important impact on our study design as some elements were left out due to the sole purpose of expecting the same results. These elements include; a) excluding a positive control group (i.e. hallucinogen or psychostimulant) and b) implementing an uninterrupted study (i.e. not implementing a break after EFV exposure to evaluate if a treatment phase is needed). The outcome of my study (addictive-like behaviour of EFV) compared to the one performed by Möller et al., differs expressively for unknown reasons. However, I do believe it is a lesson to always implement the necessary elements and to not only rely on previous work.
3. While performing my experimental work in the Vivarium I noted how the animals exposed to the EFV became more anxious, especially when placed into new cages. The rats would immediately hide in the polyvinyl chloride pipes or underneath the paper towels. These animals had the opposite behaviour of those only exposed to a vehicle, which would start to play and explore immediately. Furthermore, the animals (exposed to EFV) also tended to be aggressive toward one another. It would be interesting to do a study that investigate the effects of EFV on anxiety as well as aggressive behaviour as it would provide further insight towards psychiatric effects induced by EFV.
4. In this study NAC proved to be a futile treatment option for the effects induced by LPS alone as well as the combination of LPS and EFV. As stated in chapter 3, I do not disregard the capacity of NAC to be a viable treatment for the induced effects, I do however suggest that a higher dosage of NAC should be tested.

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## ADDENDUM A

### ETHICS APPROVAL LETTER



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26 July 2018

Dear Dr Möller- Wolmarans

#### REVIEWER FEEDBACK AFTER THE ANIMCARE COMMITTEE MEETING

Ethics number: NWU-00162-18-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:** Evaluating the addictive properties of efavirenz: risk, resilience and the role for redox-inflammatory pathways

**Study leader:** Dr M Möller- Wolmarans

**Student:** Ms C Pieters- 25056522

**Application type:** Single study

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

The abovementioned application has been reviewed and discussed by the AnimCare committee, Faculty of Health Sciences, North-West University at a meeting held on/to be held on 26/07/2018.

**AnimCare Decision category:**

Approved	X	Upon receipt of the electronic copy of your full final application and any further documents as required by the HREC for approval, your study, it will be approved by the chairperson and a letter of approval issued.
Minor changes required for approval		Provided your amended application is accepted by the reviewers, your application will be approved by the chairperson, upon receipt of the electronic copy of your full final application and any further documents as required by the HREC for approval, after which a letter of approval will be issued.
Several changes required for approval		Provided your amended application is accepted by the reviewers, your application will be approved by the chairperson upon receipt of the electronic copy of your full final application and any further documents as required by the HREC for approval, after which a letter of approval will be issued.
Deferred		Your proposed study and ethics application requires major revisions that necessitates a second full review by the AnimCare committee. Your study might need to be resubmitted to the scientific committee if indicated below. Please revisit the application and attend to the comments provided.
Disapproved		Your proposed study and ethics application requires major scientific and ethical revisions that necessitate a second full

		review by the AnimCare committee. Your study will need to be resubmitted to the scientific committee. Please revisit the application and attend to the comments below.
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Attached find three reviewers' reports that indicate the requested changes required for your application in order to adhere to the legal requirements as specified in Ethics in Health Research: Principles, Processes and Structures (2<sup>nd</sup> ed., 2015) and the South African National Standard (SANS) document 10386:2008 entitled, "The care and use of animals for scientific purposes". It is requested that the applicants please address all requested changes as indicated in the aforementioned reviewers' reports. Once the applicants are ready to submit the rebuttal, it is requested that they send it to [Ethics-AnimCare@nwu.ac.za](mailto:Ethics-AnimCare@nwu.ac.za) and include the following:

- a. A rebuttal letter, with a specific subject title indicating "Rebuttal for approval: NWU-XXXXX-XX-XX", indicating *what* changes have been made and *where* the changes were made (referring to documents and pages) to address the requested changes
- b. All associated application documents should be attached with the changes made in the documents, **highlighted in yellow** (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 the nature of the submission e.g. "Rebuttal for approval: NWU-XXXXX-XX-XX". The e-mail should indicate the nature of the document being sent. If the changes were minimal or several, the submission will be handled via the expedited process. If the application was deferred or disapproved, it will be handled by the HREC during a meeting, following receipt of your changes, with the necessary scientific committee approval.

After all corrections have been reviewed and sufficiently addressed, this study will receive approval following the electronic submission of the subsequent documentation (as determined during the review process by the AnimCare committee):

- a. Please provide the AnimCare Committee with corrected electronic copies of the full application documents (all the documents e.g. cover letter, executive summary, proposal, application form, etc. without any track changes/highlights) with the required signatures.

All documents of the application, as indicated above, should be electronically submitted to [Ethics-AnimCare@nwu.ac.za](mailto:Ethics-AnimCare@nwu.ac.za), for review BEFORE approval can be provided, with a cover letter with a specific subject title indicating "Submission of Final Version: NWU-XXXXX-XX-XX". The cover letter should include the title of the approved study, the names of the researchers involved, that the documents are being submitted after corrections, in order to obtain approval by the AnimCare committee, the nature of the documents and any further explanation to clarify the submission.

Please ensure that a full set of the application documents is included with the rebuttal letter. We wish you well with this process and you are welcome to contact us should you have any queries or comments.

Yours sincerely



Prof Christiaan B Brink  
Chair: AnimCare



Prof Minrie Greeff  
Head: Ethics Office

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## **ADDENDUM B**

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### **PRE-NATAL AND POST-NATAL EXPERIMENTAL PROTOCOLS**

**Addendum B serves as a comprehensive explanation of the methodologies followed during the pre-natal phase as well as all behavioural analyses performed throughout the post-natal phase of the study.**

Pre-natal protocol – Maternal infection

1. Saline and lipopolysaccharide (LPS) administration.

Post-natal protocol – behavioural analysis

1. Conditioned place preference (CPP)
2. Locomotor activity
3. Pre-pulse inhibition (PPI)
4. Euthanasia

#### **B.1 Pre-natal protocol**

##### **B.1.1 Introduction**

Over the last three decades numerous pre-clinical and clinical studies have demonstrated a connection between maternal infection and neurodevelopmental disorders expressed later in life (Flinkkilä et al., 2016). This connection is not subjected to the type of pathogen exposed to, but rather the subsequent maternal immune activation (MIA) and inflammation that occurs within the foetus (Guma et al., 2019). Therefore, an immune activator such as LPS (Dowling and Mansell, 2016) can be used to induce MIA within experimental animals (Moller et al., 2015). The offspring from MIA models will subsequently display behavioural and neurochemical alterations similar to alterations observed in schizophrenia (SCZ) patients (Knuesel et al., 2014, Meyer, 2014). The role of maternal infection in this study was primarily to induce a neurodevelopmental model of SCZ as previously validated in our laboratory (Swanepoel et al., 2018). Therefore, the model not only enabled us to investigate pre-natal inflammation as a contributory factor towards mental disorders later in life, but it also allowed us to evaluate how predisposed subjects would react to sub-acute EFV exposure.

##### **B.1.2 Materials**

- Vehicle (0.2 ml/day)

The vehicle consisted of isotonic saline (SAL) (0.9% sodium chloride; NaCl) solution.

- LPS (100 µg/kg)

LPS derived from *Escherichia coli* (Sigma-Aldrich, Johannesburg, South Africa) was dissolved in isotonic SAL (0.9% NaCl) solution (Swanepoel et al., 2018).

- Injections

BD Micro-Fine™ *Plus* sterile syringes (1 ml) with a 30-gauge needle (8 mm) was used to inject the animals subcutaneously (SC).

### **B.1.3 Methods**

To establish the pre-natal phase of this study, Sprague-Dawley (SD) breeding pairs were obtained and subjected to a breeding program as set out by the Vivarium (North-West University; NWU). The SD breeding pairs were housed under identical conditions: one pair per individual cage, temperature ( $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ), humidity ( $50 \pm 10\%$ ), white light (350-400 lux), 12h light/dark cycle (lights on 06h00 to 18h00) and food (pellets) and water were provided *ad libitum* (Mouton et al., 2016). These cages had grid flooring with a plastic tray placed underneath. This enabled the researcher and laboratory technicians to identify the presence of vaginal plugs, which served as a confirmation of successful mating and pregnancy (Tain et al., 2018). The days that the vaginal plugs were identified, were marked as gestational day (GD) 0 for each dam, and the study was planned accordingly. A few days after conception, the females were each placed in a ventilated cage (230(h) x 380(w) x 380(l) mm) with corncob bedding and environmental enrichment (such as shredded paper for nesting purposes and a plastic polyvinyl chloride tube that can be used for playing, climbing, resting and hiding), temperature ( $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ), humidity ( $50 \pm 10\%$ ), white light (350-400 lux), 12h light/dark cycle (lights on 06h00 to 18h00) and food (pellets) and water were provided *ad libitum* (Mouton et al., 2016). The pregnant dams were exposed to either vehicle (SAL) or LPS (100  $\mu\text{g}/\text{kg}$ ) injections (SC) between 08h00 and 10h00 on GD 15-16 (Swanepoel et al., 2018). The SC injections were performed as set out by the standard operating procedure (SOP) of the Vivarium (SOP\_Viv\_Anim 31). GD 15-16 of rats correlates with the late first trimester of human pregnancy (Aguilar-Valles and Luheshi, 2011) and the maternal exposure period when offspring are vulnerable to develop a predisposition to psychosis-like behaviour later in life (Arsenault et al., 2014, Harvey and Boksa, 2012). The day the offspring were born was designated as post-natal day (PND) 0. Offspring were kept with their biological mothers until PND 21. On PND 21 all male rats were weaned from their mothers and placed into new cages (as described above). These rats were later randomly divided into eight exposure groups which were used from PND 48 for behavioural-, peripheral and neurochemical analyses. These eight groups were as follows:

1. SALINE-OLIVE OIL-SALINE (SAL-OO-SAL)
2. SALINE-OLIVE OIL-N-ACETYLCYSTEINE (NAC) (SAL-OO-NAC)
3. SALINE-EFAVIRENZ (EFV)-SALINE (SAL-EFV-SAL)
4. SALINE-EFV-NAC (SAL-EFV-NAC)

5. LPS-OLIVE-OIL-SALINE (LPS-OO-SAL)
6. LPS-OLIVE OIL-NAC (LPS-OO-NAC)
7. LPS-EFV-SALINE (LPS-EFV-SAL)
8. LPS-EFV-NAC (LPS-EFV-NAC)

The animals were handled on a daily basis before the study commenced to familiarize the animals with human interaction and to minimize any additional handling stress that could have occurred during the study. All female offspring born from the pregnant dams were euthanised accordingly on PND 21(as discussed in chapter 1). For a detailed layout of the pre-natal phase of this study, please see table 1. Table 1 indicates all important dates as well as the saline and LPS dosages administered to each pregnant dam.

**Table 1:** Sprague-Dawley (SD) female rats – Pre-natal exposure to either saline (SAL) or lipopolysaccharide (LPS).

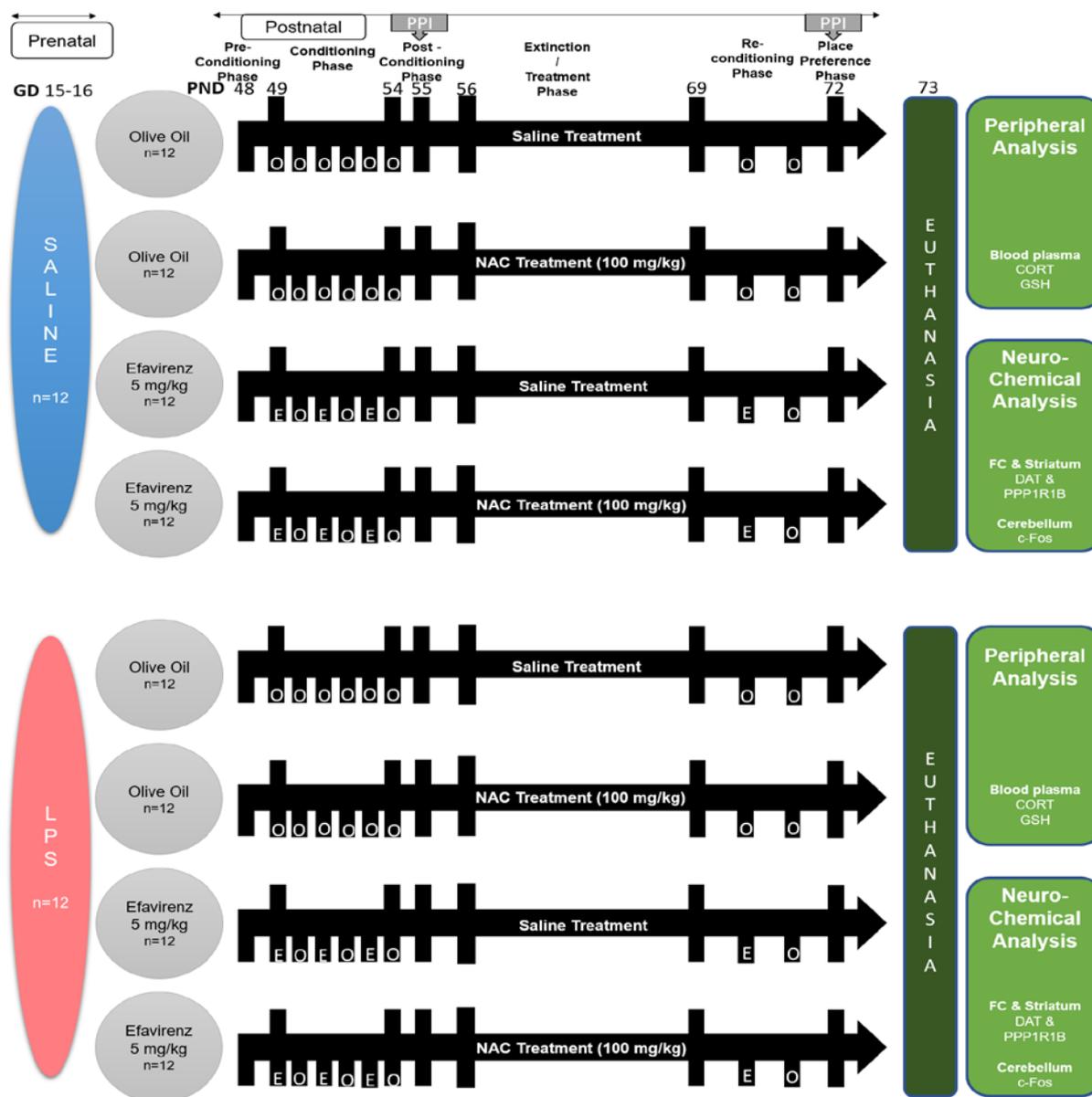
<b>PRE-NATAL EXPOSURE TO SAL</b>									
<b>SD FEMALE</b>	<b>GD 0</b>	<b>GD 15</b>	<b>GD 15 DOSE</b>	<b>DG 16</b>	<b>GD 16 DOSE</b>	<b>MALE OFFSPRING</b>	<b>PND 0</b>	<b>PND 21</b>	<b>PND 48</b>
1	28/08/2018	12/09/2018	0.2 ml	13/09/2018	0.2 ml	4	20/09/2018	11/10/2018	07/11/2018
2	28/08/2018	12/09/2018	0.2 ml	13/09/2018	0.2 ml	7	18/09/2018	09/10/2018	05/11/2018
3	29/08/2018	13/09/2018	0.2 ml	14/09/2018	0.2 ml	11	19/09/2018	10/10/2018	06/11/2018
4	30/08/2018	14/09/2018	0.2 ml	15/19/2018	0.2 ml	8	21/09/2018	12/10/2018	08/11/2018
5	02/09/2018	17/09/2018	0.2 ml	18/09/2018	0.2 ml	3	23/09/2018	14/10/2018	10/11/2018
6	15/09/2018	01/10/2018	0.2 ml	02/10/2018	0.2 ml	8	08/10/2018	29/10/2018	25/11/2018
7	27/12/2018	11/01/2019	0.2 ml	12/01/2019	0.2 ml	3	16/01/2019	06/02/2018	05/03/2019
8	27/12/2018	11/01/2019	0.2 ml	12/01/2019	0.2 ml	9	16/01/2019	06/02/2019	05/03/2019
9	No conception occurred – Rats were removed from the study								
10	No conception occurred – Rats were removed from the study								
11	No conception occurred – Rats were removed from the study								
12	No conception occurred – Rats were removed from the study								
In this study only 48 of the offspring were used, the excess rats were outsourced to other studies.									
<b>PRE-NATAL EXPOSURE TO LPS</b>									
<b>SD FEMALE</b>	<b>GD 0</b>	<b>GD 15</b>	<b>GD 15 DOSE</b>	<b>DG 16</b>	<b>GD 16 DOSE</b>	<b>MALE OFFSPRING</b>	<b>PND 0</b>	<b>PND 21</b>	<b>PND 48</b>
1	27/12/2018	11/01/2019	0.16 ml	12/01/2019	0.16 ml	7	16/11/2019	06/02/2019	05/03/2019
2	27/12/2018	11/01/2019	0.13 ml	12/01/2019	0.13 ml	4	16/11/2019	06/02/2019	05/03/2019
3	29/12/2018	13/01/2019	0.15 ml	14/01/2019	0.15 ml	7	20/01/2019	10/02/2019	09/03/2019
4	29/12/2018	13/01/2019	0.13 ml	14/01/2019	0.13 ml	7	20/01/2019	10/02/2019	09/03/2019
5	30/12/2018	14/01/2019	0.16 ml	15/01/2019	0.16 ml	4	20/01/2019	10/02/2019	09/03/2019
6	30/12/2018	14/01/2019	0.12 ml	15/01/2019	0.12 ml	5	22/01/2019	12/02/2019	11/03/2019

*Addendum B: Pre-natal and post-natal experimental protocols*

7	31/12/2018	15/01/2019	0.15 ml	16/01/2019	0.16 ml	2	21/01/2019	11/02/2019	10/03/2019
8	31/12/2018	15/01/2019	0.12 ml	16/01/2019	0.11 ml	3	21/01/2019	11/02/2019	10/03/2019
9	31/12/2018	15/01/2019	0.13ml	16/01/2019	0.14 ml	9	21/01/2019	11/02/2019	10/03/2019
10	No conception occurred – Rats were removed from the study								
11	No conception occurred – Rats were removed from the study								
12	No conception occurred – Rats were removed from the study								

## B.2 Post-natal protocol

The post-natal phase of the study was divided into six sub-phases, during which behavioural testing (CPP, locomotor activity and PPI) was performed throughout (see figure 1). All behavioural testing was performed according to previously reported methods (Möller et al., 2018, Barbosa-Méndez et al., 2018, Möller et al., 2013a) during the light cycle. For a detailed overview of how the post-natal protocol was executed for each animal, see tables 2 and 3, which appear after the discussion of all behavioural tests.



**Figure 1:** Graphical illustration of study design. Abbreviations: PPI– Pre-pulse inhibition, GD– Gestational day, PND- Post-natal day, E- Efavirenz, O- Pharmaceutical grade olive oil, GSH- Glutathione, FC- Frontal cortex, DAT- Dopamine transporters and PPP1R1B- Phosphoprotein phosphatase-1 regulatory subunit 1B

## *Materials*

- Vehicle (0.2 ml/day)

The vehicle was pharmaceutical grade olive oil (OO).

- EFV (5 mg/kg)

EFV was dissolved in a OO solution (Möller et al., 2018).

- Injections

Terumo sterile syringes (1 ml) with a 23-gauge needle (1 inch) was used to inject the animals intraperitoneally (IP).

- Vehicle (0.2 ml/day)

The vehicle consisted of isotonic SAL (0.9% NaCl) solution.

- NAC (100 mg/kg)

NAC was dissolved in a isotonic SAL (0.9% NaCl) solution (Möller et al., 2013b). The constituted NAC solution was buffered with sodium hydroxide and 1 M glacial acetic acid (pH6) (Möller et al., 2013b).

- Injections

BD Micro-Fine™ *Plus* sterile syringes (1 ml) with a 30-gauge needle (8 mm) was used to inject the animals SC.

### **B.2.1 CPP**

#### **B.2.1.1 Introduction**

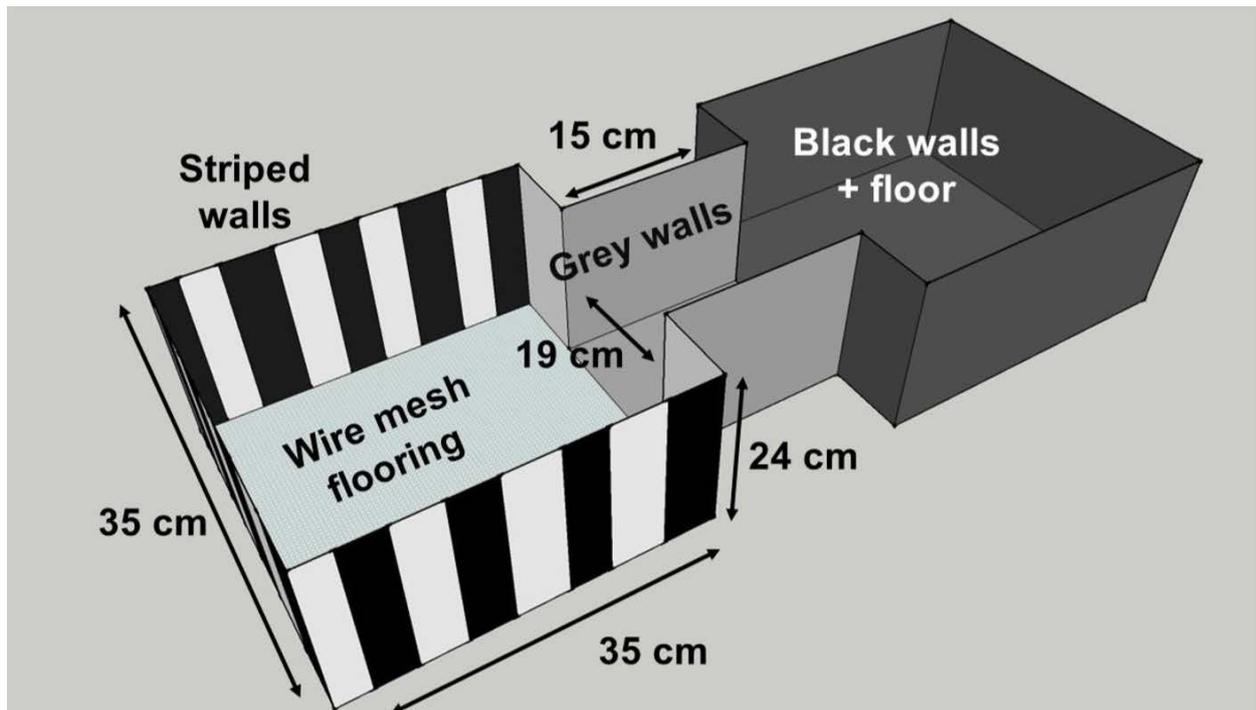
The CPP test is an appropriate high-throughput method (Smith et al., 2016) to study addiction in experimental animals (Tzschentke, 2007). This test enables the assessment of aversive- or reinforcing/rewarding properties of pharmacological substances (Lammel et al., 2012, Smith et al., 2016). In this study the CPP test was performed over the six sub-phases as indicated in figure 1.

#### **B.2.1.2 Apparatus**

##### *Apparatus*

The CPP test was performed in an apparatus made of Plexiglass comprising of three compartments, each separated by guillotine doors (Möller et al., 2018). The two larger compartments (24 x 35 cm) were separated by a much smaller central compartment (15.5 x 19.5 cm), which was only used during the pre-, post-, re-conditioning and place preference testing phases (Mueller and Stewart, 2000, Barbosa-Méndez et al., 2018). The first large outer compartment was distinguished by a wire mesh floor and black and white striped walls and the second large outer compartment by smooth Plexiglas flooring and black walls (Möller et al., 2018).

The small central compartment was designed to be neutral, with grey walls and flooring (Möller et al., 2018). For a visual representation of the CPP apparatus, see figure 2.



**Figure 2:** Visual representation of the conditioned place preference (CPP) apparatus used in our laboratory.

CPP behaviour was recorded under dim white light (30 lux) with a digital video camera, as previously validated (Möller et al., 2018). CPP behaviour was blindly scored by EthoVision© XT software (Noldus Information Technology, Wageningen, Netherlands) as the time (seconds; s) spent in each compartment.

### **B.2.1.3 Method**

The CPP behavioural test was divided into six sub-phases:

#### Phase 1: Pre-conditioning phase

The pre-conditioning phase was a basic habituation phase, and was incorporated to establish the preference of each animal. During this phase, the rats (now PND 48) were placed in the small central compartment (Barbosa-Méndez et al., 2018) and left to wander about between the compartments for 20 minutes (min) as both guillotine doors were removed (Möller et al., 2018). This allowed the rats to develop a preference for one chamber (Prus et al., 2009). The time spent in each chamber was an indication of preference, and the least preferred chamber served as the chamber in which the drug was administered to the animal (Prus et al., 2009). The aim of this

phase was to ensure that novelty was eliminated as a potential experimental variable (Prus et al., 2009).

#### Phase 2: Conditioning phase

In this phase, the animals underwent intermittent conditioning sessions and pseudo-conditioning sessions, depending on the relevant drug's unique rewarding properties (Huston et al., 2013, Prus et al., 2009, Barbosa-Méndez et al., 2018). The conditioning session involved the drug (EFV) being administered IP to the each animal followed by placing them into their respective least-preferred chamber (Prus et al., 2009), an hour after EFV exposure. The animals were then left in the chamber for a specified period of 20 min (Möller et al., 2018). This procedure was performed on PNDs 49,51 and 53. The IP injections were performed as set out by the SOP of the Vivarium (SOP\_Viv\_Anim 3). The pseudo-conditioning session occurred within the chamber the each animal preferred the most and where the animal received a placebo (vehicle) treatment, in this case OO (Prus et al., 2009). The vehicle was also administered an hour before placing the animal back into the apparatus. Once again, the animals were left in the chamber for a specified period of 20 min (Möller et al., 2018). This procedure was applied on PNDs 50,52 and 54. The pseudo-conditioning session was essential to eliminate the emotional effects associated with the drug administration process (Huston et al., 2013). After each conditioning session, rats were placed back into their home cages (Barbosa-Méndez et al., 2018).

#### Phase 3: Post-conditioning test

In this phase, the rats (now PND 55) were placed back into the small grey compartment and allowed to wander about for 20 min between all compartments as both guillotine doors were removed (Bardo et al., 1995, Barbosa-Méndez et al., 2018, Möller et al., 2018). The time each animal spent in a specific chamber was determined again and compared to the times measured during the pre-conditioning phase (Bardo et al., 1995, Huston et al., 2013). If an animal spent more time in the chamber associated with drug administration, it can be concluded that the drug showed rewarding properties (Huston et al., 2013, Gatch et al., 2013). However, the opposite is also true, if the animal spent more time in the chamber associated with the placebo treatment, the drug demonstrated aversive properties (Huston et al., 2013, Gatch et al., 2013).

#### Phase 4: Extinction / Treatment phase

During the extinction/treatment phase the rats (now PND 56-69) received a daily SC injection of either SAL or NAC (depending on the group they are allocated to) after which they were returned to their home cages.

#### Phase 5: Re-conditioning phase

In this phase the animals (now PND 70-71) received an EFV injection (PND 70) and were placed back into the drug-paired compartment (an hour after EFV exposure) followed by an OO injection (PND 71) in the non-drug paired compartment. The rats were left to roam freely for 20 minutes (Barbosa-Méndez et al., 2018).

#### Phase 6: Place preference testing phase

In the last phase (PND 72) of the experiment, animals were placed into the small grey compartment of the apparatus in a drug-free state and left to roam freely for 20 min between all three compartments, since both guillotine doors were removed at this time. Thereafter the amount of time spent in each chamber was determined, again using Ethovision© software and compared to the pre-conditioning phase data (Barbosa-Méndez et al., 2018).

Formulas used to calculate CPP:

CPP before NAC treatment: *Time spent in drug-paired compartment during post-conditioning phase (s) – Time spent in drug-paired compartment during pre-conditioning phase (s).*

CPP after NAC treatment: *Time spent in drug-paired compartment during place preference phase (s) – Time spent in drug-paired compartment during pre-conditioning phase (s).*

## **B.2.2 Locomotor activity**

### **B.2.2.1 Introduction**

The evaluation of locomotor activity in rats provides significant insight towards addictive- and SCZ-like behaviours (Jones et al., 2011). In this study locomotor activity was measured twice, first after sub-acute exposure to EFV and secondly after the chronic NAC treatment was implemented.

### **B.2.2.2 Apparatus**

Locomotor activity was measured within the CPP apparatus while conducting the CPP behavioural test. Therefore, the apparatus used are indicated in figure 2. Locomotor activity was scored as per the conditions of the CPP test, thus also under dim white light (30 lux) with a digital video camera and blindly scored by EthoVision© XT software (Noldus Information Technology, Wageningen, Netherlands) as the total distance moved (cm) by each subject during each of the aforementioned testing phases.

### **B.2.2.3 Method**

Locomotor activity was scored on PND 55 and 72 during the post-conditioning- and place-preference phase when the rats were subjected to CPP testing. Thus, the locomotor activity (distance moved) was measured in the CPP apparatus when the animals had free access to all three compartments for 20 min.

### **B.2.3 PPI**

#### **B.2.3.1 Introduction**

PPI of the startle response is widely accepted as a dependable quantitative method to analyse sensorimotor gating which is altered in numerous mental disorders (Swerdlow et al., 2008, Braff et al., 1978). PPI is considered as the “interface of cognition and psychosis” (Desbonnet et al., 2009) and therefore, the PPI was applied to evaluate psychotic-like behaviour.

#### **B.2.3.2 Apparatus**

The apparatus used in this study is illustrated in figure 3 (SRLAB, San Diego Instruments, San Diego, USA) which is applicable to test PPI as well as startle habituation. The startle chamber contained a slotted cylindrical animal enclosure made of clear plexiglass in which the animals were placed (constrained) in for the duration of the test. This enclosure was placed horizontally on a small mobile platform which rested on a solid base inside the startle chamber. This platform allowed the measurement of a startle after each pulse or stimulus. A high-frequency loudspeaker was fixed above the animal enclosure. In our laboratory we have two PPI systems, therefore allowing for the simultaneous testing of two animals. All experimental data was obtained electronically via SR-LAB specialized software output.



**Figure 3:** Visual representation of the pre-pulse inhibition (PPI) apparatus used in our laboratory.

### **B.2.2.3 Method**

PPI tests were performed on PND 55 and PND 72 of the post-natal phase, in the same groups of animals approximately an hour after the CPP testing, according to a method previously set out (Möller et al., 2013a).

When performing the PPI test, each male SD rat was placed within a ventilated sound-attenuated startle chambers (Figure 3) and habituated with 65 dB background white noise for 5 minutes. This background white noise was present throughout the entire PPI test session. The lights inside the chambers also remained on for the duration of the PPI test.

100 PPI trials were performed, with average intervals of 25 seconds. These PPI trials included a 20ms non-startling pre-pulse (72, 76, 80 or 84 dB) followed by a single 40ms startle stimulus (120 dB) 80ms later. PPI was performed in 4 blocks:

- Block 1: first ten pulse-alone stimuli,
- Block 2: 20 pulse-alone stimuli and pre-pulse stimuli,
- Block 3: 20 pulse-alone stimuli and pre-pulse stimuli and
- Block 4: last ten pulse-alone stimuli.

These blocks served as a measure of the mean startle amplitude, which indicate the rat's habituation towards the re-occurring startling stimuli (Van den Buuse and Eikelis, 2001). The formula used to calculate PPI is as follows (Tunstall et al., 2009);

$$PPI = 100 - \left( 100 \times \left( \frac{\text{mean}(\text{pulse alone}) - \text{mean}(\text{prepulse})}{\text{mean}(\text{pulse alone})} \right) \right)$$

After obtaining the %PPI data across all trials at 72, 76, 80 and 84 Db pre-pulses, an average %PPI was calculated (see formula below).

$$AVERAGE \%PPI = \frac{\%PPI(72dB) + \%PPI(76dB) + \%PPI(80dB) + \%PPI(84dB)}{4}$$

After the PPI test, animals were placed back into their home cages, and a two-hour post-test evaluation was performed to monitor (via a study specific NWU Vivarium monitoring sheet) any potential excessive distress or behavioural abnormalities. In this study, no animal demonstrated noteworthy distress after PPI testing.

## **B. 2.4 Euthanasia protocol**

After all behavioural testing were completed, the animals were euthanised via decapitation on PND 73. This was done between 08h00 and 10h00 according to the SOP set out by the Vivarium (SOP\_Viv\_Anim 1).

Addendum B: Pre-natal and post-natal experimental protocols

**Table 2:** Detailed outlay of experimental procedures from November – December 2018. The animals used (November-December) were prenatally exposed to saline.

		November 2018						
DATE	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	
PND ACCORDING TO DATE OF BIRTH	5 48	6 49 48 C1R1-ND-CPP C1R2-ND-CPP	7 50 49 48 C1R1-O-CPP C1R2-O-CPP  C1R4-ND-CPP	8 51 50 49 48 C1R1-O-CPP C1R2-O-CPP C1R3-ND-CPP C1R4-O-CPP	9 52 51 50 49 C1R1-O-CPP C1R2-O-CPP C1R3-O-CPP C1R4-O-CPP	10 53 52 51 50 48 C1R1-O-CPP C1R2-O-CPP C1R3-O-CPP C1R4-O-CPP	11 54 53 52 51 49 C1R1-O-CPP C1R2-O-CPP C1R3-O-CPP C1R4-O-CPP	
	C2R1-ND-CPP	C2R1-O-CPP  C2R3-ND-CPP	C2R1-O-CPP  C2R3-O-CPP	C2R1-O-CPP C2R2-ND-CPP C2R3-O-CPP	C2R1-O-CPP C2R2-O-CPP C2R3-O-CPP	C2R1-O-CPP C2R2-O-CPP C2R3-O-CPP C2R4-ND-CPP	C2R1-O-CPP C2R2-O-CPP C2R3-O-CPP C2R4-O-CPP	
	C3R1-ND-CPP	C3R1-O-CPP  C3R3-ND-CPP	C3R1-O-CPP  C3R3-O-CPP	C3R1-O-CPP  C3R3-O-CPP C3R4-ND-CPP	C3R1-O-CPP  C3R3-O-CPP C3R4-O-CPP	C3R1-O-CPP C3R2-ND-CPP C3R3-O-CPP C3R4-O-CPP	C3R1-O-CPP C3R2-O-CPP C3R3-O-CPP C3R4-O-CPP	
		C4R1-ND-CPP C4R3-ND-CPP	C4R1-O-CPP C4R3-O-CPP	C4R1-O-CPP C4R3-O-CPP  C4R4-ND-CPP	C4R1-O-CPP C4R3-O-CPP  C4R4-O-CPP	C4R1-O-CPP C4R2-ND-CPP C4R3-O-CPP C4R4-O-CPP	C4R1-O-CPP C4R2-O-CPP C4R3-O-CPP C4R4-O-CPP	
	C5R1-ND-CPP	C5R1-O-CPP  C5R4-ND-CPP	C5R1-O-CPP  C5R4-O-CPP C5R3-ND-CPP	C5R1-O-CPP C5R2-ND-CPP C5R3-O-CPP C5R4-O-CPP	C5R1-O-CPP C5R2-O-CPP C5R3-O-CPP C5R4-O-CPP	C5R1-O-CPP C5R2-O-CPP C5R3-O-CPP C5R4-O-CPP	C5R1-O-CPP C5R2-O-CPP C5R3-O-CPP C5R4-O-CPP	
	C6R2-ND-CPP	C6R1-ND-CPP C6R2-O-CPP	C6R1-O-CPP C6R2-O-CPP C6R3-ND-CPP	C6R1-O-CPP C6R2-O-CPP C6R3-O-CPP	C6R1-O-CPP C6R2-O-CPP C6R3-O-CPP	C6R1-O-CPP C6R2-O-CPP C6R3-O-CPP	C6R1-O-CPP C6R2-O-CPP C6R3-O-CPP	
	C6R4-ND-CPP	C6R4-O-CPP	C6R4-O-CPP	C6R4-O-CPP	C6R4-O-CPP	C6R4-O-CPP	C6R4-O-CPP	
	C7R1-ND-CPP	C7R1-E-CPP	C7R1-O-CPP	C7R1-E-CPP	C7R1-O-CPP	C7R1-E-CPP	C7R1-O-CPP	

Addendum B: Pre-natal and post-natal experimental protocols

	<b>C7R2-ND-CPP</b>	<b>C7R2-E-CPP</b>	<b>C7R2-O-CPP</b> <b>C7R3-ND-CPP</b> <b>C7R4-ND-CPP</b>	<b>C7R2-E-CPP</b> <b>C7R3-E-CPP</b> <b>C7R4-E-CPP</b>	<b>C7R2-O-CPP</b> <b>C7R3-O-CPP</b> <b>C7R4-O-CPP</b>	<b>C7R2-E-CPP</b> <b>C7R3-E-CPP</b> <b>C7R4-E-CPP</b>
<b>C8R2-ND-CPP</b>	<b>C8R1-ND-CPP</b> <b>C8R2-E-CPP</b>  <b>C8R4-ND-CPP</b>	<b>C8R1-E-CPP</b> <b>C8R2-O-CPP</b> <b>C8R3-ND-CPP</b> <b>C8R4-E-CPP</b>	<b>C8R1-O-CPP</b> <b>C8R2-E-CPP</b> <b>C8R3-E-CPP</b> <b>C8R4-O-CPP</b>  <b>C9R1-ND-CPP</b>	<b>C8R1-E-CPP</b> <b>C8R2-O-CPP</b> <b>C8R3-O-CPP</b> <b>C8R4-E-CPP</b>  <b>C9R1-E-CPP</b>	<b>C8R1-O-CPP</b> <b>C8R2-E-CPP</b> <b>C8R3-E-CPP</b> <b>C8R4-O-CPP</b>  <b>C9R1-O-CPP</b>	<b>C8R1-E-CPP</b> <b>C8R2-O-CPP</b> <b>C8R3-O-CPP</b> <b>C8R4-E-CPP</b>  <b>C9R1-E-CPP</b>
<b>12 55 54 53 52 50</b> <b>C1R1-O-CPP</b> <b>C1R2-O-CPP</b> <b>C1R3-O-CPP</b> <b>C1R4-O-CPP</b>	<b>13 56 55 54 53 51</b> <b>C1R1-ND-C/PPI</b> <b>C1R2-ND-C/PPI</b> <b>C1R3-O-CPP</b> <b>C1R4-O-CPP</b>	<b>14 57 56 55 54 52</b> <b>C1R1-SALINE</b> <b>C1R2-SALINE</b> <b>C1R3-O-CPP</b> <b>C1R4-ND-C/PPI</b>	<b>15 58 57 56 55 53</b> <b>C1R1-SALINE</b> <b>C1R2-SALINE</b> <b>C1R3-ND-C/PPI</b> <b>C1R4-SALINE</b>	<b>16 59 58 57 56 54</b> <b>C1R1-SALINE</b> <b>C1R2-SALINE</b> <b>C1R3-SALINE</b> <b>C1R4-SALINE</b>	<b>17 60 59 58 57 55</b> <b>C1R1-SALINE</b> <b>C1R2-SALINE</b> <b>C1R3-SALINE</b> <b>C1R4-SALINE</b>	<b>18 61 60 59 58 56</b> <b>C1R1-SALINE</b> <b>C1R2-SALINE</b> <b>C1R3-SALINE</b> <b>C1R4-SALINE</b>
<b>C2R1-ND-C/PPI</b> <b>C2R2-O-CPP</b> <b>C2R3-O-CPP</b> <b>C2R4-O-CPP</b>	<b>C2R1-SALINE</b> <b>C2R2-O-CPP</b> <b>C2R3-ND-C/PPI</b> <b>C2R4-O-CPP</b>	<b>C2R1-SALINE</b> <b>C2R2-O-CPP</b> <b>C2R3-SALINE</b> <b>C2R4-O-CPP</b>	<b>C2R1-SALINE</b> <b>C2R2-ND-C/PPI</b> <b>C2R3-SALINE</b> <b>C2R4-O-CPP</b>	<b>C2R1-SALINE</b> <b>C2R2-SALINE</b> <b>C2R3-SALINE</b> <b>C2R4-O-CPP</b>	<b>C2R1-SALINE</b> <b>C2R2-SALINE</b> <b>C2R3-SALINE</b> <b>C2R4-ND-C/PPI</b>	<b>C2R1-SALINE</b> <b>C2R2-SALINE</b> <b>C2R3-SALINE</b> <b>C2R4-SALINE</b>
<b>C3R1-ND-C/PPI</b> <b>C3R2-O-CPP</b> <b>C3R3-O-CPP</b> <b>C3R4-O-CPP</b>	<b>C3R1-SALINE</b> <b>C3R2-O-CPP</b> <b>C3R3-ND-C/PPI</b> <b>C3R4-O-CPP</b>	<b>C3R1-SALINE</b> <b>C3R2-O-CPP</b> <b>C3R3-SALINE</b> <b>C3R4-O-CPP</b>	<b>C3R1-SALINE</b> <b>C3R2-O-CPP</b> <b>C3R3-SALINE</b> <b>C3R4-ND-C/PPI</b>	<b>C3R1-SALINE</b> <b>C3R2-O-CPP</b> <b>C3R3-SALINE</b> <b>C3R4-SALINE</b>	<b>C3R1-SALINE</b> <b>C3R2-ND-C/PPI</b> <b>C3R3-SALINE</b> <b>C3R4-SALINE</b>	<b>C3R1-SALINE</b> <b>C3R2-SALINE</b> <b>C3R3-SALINE</b> <b>C3R4-SALINE</b>
<b>C4R1-O-CPP</b> <b>C4R2-O-CPP</b> <b>C4R3-O-CPP</b> <b>C4R4-O-CPP</b>	<b>C4R1-ND-C/PPI</b> <b>C4R2-O-CPP</b> <b>C4R3-ND-C/PPI</b> <b>C4R4-O-CPP</b>	<b>C4R1-NAC</b> <b>C4R2-O-CPP</b> <b>C4R3-NAC</b> <b>C4R4-O-CPP</b>	<b>C4R1-NAC</b> <b>C4R2-O-CPP</b> <b>C4R3-NAC</b> <b>C4R4-ND-C/PPI</b>	<b>C4R1-NAC</b> <b>C4R2-O-CPP</b> <b>C4R3-NAC</b> <b>C4R4-NAC</b>	<b>C4R1-NAC</b> <b>C4R2-ND-C/PPI</b> <b>C4R3-NAC</b> <b>C4R4-NAC</b>	<b>C4R1-NAC</b> <b>C4R2-NAC</b> <b>C4R3-NAC</b> <b>C4R4-NAC</b>
<b>C5R1-ND-C/PPI</b> <b>C5R2-O-CPP</b> <b>C5R3-O-CPP</b> <b>C5R4-O-CPP</b>	<b>C5R1-NAC</b> <b>C5R2-O-CPP</b> <b>C5R3-O-CPP</b> <b>C5R4-ND-C/PPI</b>	<b>C5R1-NAC</b> <b>C5R2-O-CPP</b> <b>C5R3-ND-C/PPI</b> <b>C5R4-NAC</b>	<b>C5R1-NAC</b> <b>C5R2-ND-C/PPI</b> <b>C5R3-NAC</b> <b>C5R4-NAC</b>	<b>C5R1-NAC</b> <b>C5R2-NAC</b> <b>C5R3-NAC</b> <b>C5R4-NAC</b>	<b>C5R1-NAC</b> <b>C5R2-NAC</b> <b>C5R3-NAC</b> <b>C5R4-NAC</b>	<b>C5R1-NAC</b> <b>C5R2-NAC</b> <b>C5R3-NAC</b> <b>C5R4-NAC</b>

Addendum B: Pre-natal and post-natal experimental protocols

<p>C6R1-O-CPP C6R2-ND-C/PPI C6R3-O-CPP C6R4-ND-C/PPI</p> <p>C7R1-ND-C/PPI C7R2-O-CPP C7R3-O-CPP C7R4-O-CPP</p> <p>C8R1-O-CPP C8R2-ND-C/PPI C8R3-E-CPP C8R4-O-CPP C9R1-O-CPP</p>	<p>C6R1-ND-C/PPI C6R2-NAC C6R3-O-CPP C6R4-NAC</p> <p>C7R1-SALINE C7R2-ND-C/PPI C7R3-E-CPP C7R4-E-CPP</p> <p>C8R1-ND-C/PPI C8R2-SALINE C8R3-O-CPP C8R4-ND-C/PPI C9R1-E-CPP</p>	<p>C6R1-NAC C6R2-NAC C6R3-ND-C/PPI C6R4-NAC</p> <p>C7R1-SALINE C7R2-SALINE C7R3-O-CPP C7R4-O-CPP</p> <p>C8R1-SALINE C8R2-SALINE C8R3-ND-C/PPI C8R4-SALINE C9R1-O-CPP</p>	<p>C6R1-NAC C6R2-NAC C6R3-NAC C6R4-NAC</p> <p>C7R1-SALINE C7R2-SALINE C7R3-ND-C/PPI C7R4-ND-C/PPI</p> <p>C8R1-SALINE C8R2-SALINE C8R3-SALINE C8R4-SALINE C9R1-ND-C/PPI</p>	<p>C6R1-NAC C6R2-NAC C6R3-NAC C6R4-NAC</p> <p>C7R1-SALINE C7R2-SALINE C7R3-SALINE C7R4-SALINE</p> <p>C8R1-SALINE C8R2-SALINE C8R3-SALINE C8R4-SALINE C9R1-SALINE</p>	<p>C6R1-NAC C6R2-NAC C6R3-NAC C6R4-NAC</p> <p>C7R1-SALINE C7R2-SALINE C7R3-SALINE C7R4-SALINE</p> <p>C8R1-SALINE C8R2-SALINE C8R3-SALINE C8R4-SALINE C9R1-SALINE</p>	
<p>19 62 61 60 59 57 C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-SALINE C2R2-SALINE C2R3-SALINE C2R4-SALINE</p> <p>C3R1-SALINE C3R2-SALINE C3R3-SALINE C3R4-SALINE</p> <p>C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC</p> <p>C5R1-NAC</p>	<p>20 63 62 61 60 58 C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-SALINE C2R2-SALINE C2R3-SALINE C2R4-SALINE</p> <p>C3R1-SALINE C3R2-SALINE C3R3-SALINE C3R4-SALINE</p> <p>C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC</p> <p>C5R1-NAC</p>	<p>21 64 63 62 61 59 C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-SALINE C2R2-SALINE C2R3-SALINE C2R4-SALINE</p> <p>C3R1-SALINE C3R2-SALINE C3R3-SALINE C3R4-SALINE</p> <p>C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC</p> <p>C5R1-NAC</p>	<p>22 65 64 63 62 60 C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-SALINE C2R2-SALINE C2R3-SALINE C2R4-SALINE</p> <p>C3R1-SALINE C3R2-SALINE C3R3-SALINE C3R4-SALINE</p> <p>C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC</p> <p>C5R1-NAC</p>	<p>23 66 65 64 63 61 C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-SALINE C2R2-SALINE C2R3-SALINE C2R4-SALINE</p> <p>C3R1-SALINE C3R2-SALINE C3R3-SALINE C3R4-SALINE</p> <p>C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC</p> <p>C5R1-NAC</p>	<p>24 67 66 65 64 62 C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-SALINE C2R2-SALINE C2R3-SALINE C2R4-SALINE</p> <p>C3R1-SALINE C3R2-SALINE C3R3-SALINE C3R4-SALINE</p> <p>C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC</p> <p>C5R1-NAC</p>	<p>25 68 67 66 65 63 48 C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-SALINE C2R2-SALINE C2R3-SALINE C2R4-SALINE</p> <p>C3R1-SALINE C3R2-SALINE C3R3-SALINE C3R4-SALINE</p> <p>C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC</p> <p>C5R1-NAC</p>

Addendum B: Pre-natal and post-natal experimental protocols

<p>C5R2-NAC C5R3-NAC C5R4-NAC</p> <p>C6R1-NAC C6R2-NAC C6R3-NAC C6R4-NAC</p> <p>C7R1-SALINE C7R2-SALINE C7R3-SALINE C7R4-SALINE</p> <p>C8R1-SALINE C8R2-SALINE C8R3-SALINE C8R4-SALINE</p> <p>C9R1-SALINE</p>	<p>C5R2-NAC C5R3-NAC C5R4-NAC</p> <p>C6R1-NAC C6R2-NAC C6R3-NAC C6R4-NAC</p> <p>C7R1-SALINE C7R2-SALINE C7R3-SALINE C7R4-SALINE</p> <p>C8R1-SALINE C8R2-SALINE C8R3-SALINE C8R4-SALINE</p> <p>C9R1-SALINE C9R2-ND-CPP C9R3-ND-CPP C9R4-ND-CPP</p> <p>C10R1-ND-CPP C10R2-ND-CPP C10R3-ND-CPP</p> <p>C11R1-ND-CPP C11R2-ND-CPP</p>					
<p>26 69 68 67 66 64 49</p> <p>C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-SALINE</p>	<p>27 70 69 68 67 65 50</p> <p>C1R1-SALINE C1R2-SALINE C1R3-SALINE C1R4-SALINE</p> <p>C2R1-O-CPP</p>	<p>28 71 70 69 68 66 51</p> <p>C1R1-O-CPP C1R2-O-CPP C1R3-SALINE C1R4-SALINE</p> <p>C2R1-O-CPP</p>	<p>29 72 71 70 69 67 52</p> <p>C1R1-O-CPP C1R2-O-CPP C1R3-SALINE C1R4-O-CPP</p> <p>C2R1-ND-C/PPI</p>	<p>30 73 72 71 70 68 53</p> <p>C1R1-ND-C/PPI C1R2-ND-C/PPI C1R3-O-CPP C1R4-O-CPP</p> <p>C2R1-EUTH</p>		

Addendum B: Pre-natal and post-natal experimental protocols

C2R2-SALINE C2R3-SALINE C2R4-SALINE	C2R2-SALINE C2R3-SALINE C2R4-SALINE	C2R2-SALINE C2R3-O-CPP C2R4-SALINE	C2R2-SALINE C2R3-O-CPP C2R4-SALINE	C2R2-O-CPP C2R3-ND-C/PPI C2R4-SALINE		
C3R1-SALINE C3R2-SALINE C3R3-SALINE C3R4-SALINE	C3R1-O-CPP C3R2-SALINE C3R3-SALINE C3R4-SALINE	C3R1-O-CPP C3R2-SALINE C3R3-O-CPP C3R4-SALINE	C3R1-ND-C/PPI C3R2-SALINE C3R3-O-CPP C3R4-SALINE	C3R1-EUTH C3R2-SALINE C3R3-ND-C/PPI C3R4-O-CPP		
C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC	C4R1-NAC C4R2-NAC C4R3-NAC C4R4-NAC	C4R1-O-CPP C4R2-NAC C4R3-O-CPP C4R4-NAC	C4R1-O-CPP C4R2-NAC C4R3-O-CPP C4R4-NAC	C4R1-ND-C/PPI C4R2-NAC C4R3-ND-C/PPI C4R4-O-CPP		
C5R1-NAC C5R2-NAC C5R3-NAC C5R4-NAC	C5R1-O-CPP C5R2-NAC C5R3-NAC C5R4-NAC	C5R1-O-CPP C5R2-NAC C5R3-NAC C5R4-O-CPP	C5R1-ND-C/PPI C5R2-NAC C5R3-O-CPP C5R4-O-CPP	C5R1-EUTH C5R2-O-CPP C5R3-O-CPP C5R4-ND-C/PPI		
C6R1-NAC C6R2-NAC C6R3-NAC C6R4-NAC	C6R1-NAC C6R2-O-CPP C6R3-NAC C6R4-O-CPP	C6R1-O-CPP C6R2-O-CPP C6R3-NAC C6R4-O-CPP	C6R1-O-CPP C6R2-ND-C/PPI C6R3-O-CPP C6R4-ND-C/PPI	C6R1-ND-C/PPI C6R2-EUTH C6R3-O-CPP C6R4-EUTH		
C7R1-SALINE C7R2-SALINE C7R3-SALINE C7R4-SALINE	C7R1-E-CPP C7R2-SALINE C7R3-SALINE C7R4-SALINE	C7R1-O-CPP C7R2-E-CPP C7R3-SALINE C7R4-SALINE	C7R1-ND-C/PPI C7R2-O-CPP C7R3-SALINE C7R4-SALINE	C7R1-EUTH C7R2-ND-C/PPI C7R3-E-CPP C7R4-E-CPP		
C8R1-SALINE C8R2-SALINE C8R3-SALINE C8R4-SALINE	C8R1-SALINE C8R2-E-CPP C8R3-SALINE C8R4-SALINE	C8R1-E-CPP C8R2-O-CPP C8R3-SALINE C8R4-E-CPP	C8R1-O-CPP C8R2-ND-C/PPI C8R3-E-CPP C8R4-O-CPP	C8R1-ND-C/PPI C8R2-EUTH C8R3-O-CPP C8R4-ND-C/PPI		
C9R1-SALINE C9R2-E-CPP C9R3-E-CPP	C9R1-SALINE C9R2-O-CPP C9R3-O-CPP	C9R1-SALINE C9R2-E-CPP C9R3-E-CPP	C9R1-SALINE C9R2-O-CPP C9R3-O-CPP	C9R1-E-CPP C9R2-E-CPP C9R3-E-CPP		

Addendum B: Pre-natal and post-natal experimental protocols

C9R4-E-CPP	C9R4-O-CPP	C9R4-E-CPP	C9R4-O-CPP	C9R4-E-CPP		
C10R1-E-CPP	C10R1-O-CPP	C10R1-E-CPP	C10R1-O-CPP	C10R1-E-CPP		
C10R2-E-CPP	C10R2-O-CPP	C10R2-E-CPP	C10R2-O-CPP	C10R2-E-CPP		
C10R3-E-CPP	C10R3-O-CPP	C10R3-E-CPP	C10R3-O-CPP	C10R3-E-CPP		
C11R1-E-CPP	C11R1-O-CPP	C11R1-E-CPP	C11R1-O-CPP	C11R1-E-CPP		
C11R2-E-CPP	C11R2-O-CPP	C11R2-E-CPP	C11R2-O-CPP	C11R2-E-CPP		
December 2018						
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
						<b>1 73 72 71 69 54</b> <b>C1R1-EUHT</b> <b>C1R2-EUTH</b> <b>C1R3-O-CPP</b> <b>C1R4-ND-C/PPI</b>  <b>C2R2-O-CPP</b> <b>C2R3-EUTH</b> <b>C2R4-SALINE</b>  <b>C3R2-SALINE</b> <b>C3R3-EUTH</b> <b>C3R4-O-CPP</b>  <b>C4R1-EUTH</b> <b>C4R2-NAC</b> <b>C4R3-EUTH</b> <b>C4R4-O-CPP</b>  <b>C5R2-O-CPP</b> <b>C5R3-ND-C/PPI</b> <b>C5R4-EUTH</b> <b>C6R1-EUTH</b> <b>C6R3-ND-C/PPI</b>  <b>C7R2-EUTH</b> <b>C7R3-O-CPP</b>

Addendum B: Pre-natal and post-natal experimental protocols

						<p>C7R4-O-CPP</p> <p>C8R1-EUTH C8R3-ND-C/PPI C8R4-EUTH</p> <p>C9R1-O-CPP C9R2-O-CPP C9R3-O-CPP C9R4-O-CPP</p> <p>C10R1-O-CPP C10R2-O-CPP C10R3-O-CPP</p> <p>C11R1-O-CPP C11R2-O-CPP</p>
<p>2 73 72 70 55</p> <p>C1R4-EUTH C1R3-ND-C/PPI</p> <p>C2R2-ND-C/PPI C2R4-O-CPP</p> <p>C3R2-O-CPP C3R4-ND-C/PPI</p> <p>C4R2-O-CPP C4R4-ND-C/PPI</p> <p>C5R2-ND-C/PPI C5R3-EUTH</p> <p>C6R3-EUTH</p> <p>C7R3-ND-C/PPI C7R4-ND-C/PPI</p>	<p>3 73 71 56</p> <p>C1R3-EUTH</p> <p>C2R2-EUTH C2R4-O-CPP</p> <p>C3R2-O-CPP C3R4-EUTH</p> <p>C4R2-O-CPP C4R4-EUTH</p> <p>C5R2-EUTH</p> <p>C7R3-EUTH C7R4-EUTH</p>	<p>4 72 57</p> <p>C2R4-ND-C/PPI</p> <p>C3R2-ND-C/PPI</p> <p>C4R2-ND-C/PPI</p>	<p>5 73 58</p> <p>C2R4-EUTH</p> <p>C3R2-EUTH</p> <p>C4R2-EUTH</p>	<p>6 59</p>	<p>7 60</p>	<p>8 61</p>

Addendum B: Pre-natal and post-natal experimental protocols

<b>C8R3-EUTH</b>						
<b>C9R1-ND-C/PPI</b> <b>C9R2-ND-C/PPI</b> <b>C9R3-ND-C/PPI</b> <b>C9R4-ND-C/PPI</b>	<b>C9R1-EUTH</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>
<b>C10R1-ND-C/PPI</b> <b>C10R2-ND-C/PPI</b> <b>C10R3-ND-C/PPI</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>
<b>C11R1-ND-C/PPI</b> <b>C11R2-ND-C/PPI</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>
<b>9 62</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>10 63</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>11 64</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>12 65</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>13 66</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>14 67</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>15 68</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>
<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>
<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-NAC</b> <b>C11R2-NAC</b>
<b>16 69</b> <b>C9R2-NAC</b> <b>C9R3-NAC</b> <b>C9R4-NAC</b>	<b>17 70</b> <b>C9R2-E-CPP</b> <b>C9R3-E-CPP</b> <b>C9R4-E-CPP</b>	<b>18 71</b> <b>C9R2-O-CPP</b> <b>C9R3-O-CPP</b> <b>C9R4-O-CPP</b>	<b>19 72</b> <b>C9R2-ND-C/PPI</b> <b>C9R3-ND-C/PPI</b> <b>C9R4-ND-C/PPI</b>	<b>20 73</b> <b>C9R2-EUTH</b> <b>C9R3-EUTH</b> <b>C9R4-EUTH</b>		
<b>C10R1-NAC</b> <b>C10R2-NAC</b> <b>C10R3-NAC</b>	<b>C10R1-E-CPP</b> <b>C10R2-E-CPP</b> <b>C10R3-E-CPP</b>	<b>C10R1-O-CPP</b> <b>C10R2-O-CPP</b> <b>C10R3-O-CPP</b>	<b>C10R1-ND-C/PPI</b> <b>C10R2-ND-C/PPI</b> <b>C10R3-ND-C/PPI</b>	<b>C10R1-EUTH</b> <b>C10R2-EUTH</b> <b>C10R3-EUTH</b>		
<b>C11R1-NAC</b> <b>C11R2-NAC</b>	<b>C11R1-E-CPP</b> <b>C11R2-E-CPP</b>	<b>C11R1-O-CPP</b> <b>C11R2-O-CPP</b>	<b>C11R1-ND-C/PPI</b> <b>C11R2-ND-C/PPI</b>	<b>C11R1-EUTH</b> <b>C11R2-EUTH</b>		

Addendum B: Pre-natal and post-natal experimental protocols

Dates of birth: 18/09/2018, 19/09/2018, 20/09/2019, 21/09/2018, 23/09/2018. Abbreviations: E- Efavirenz, O- Olive oil, ND- No drug administered, CPP- Conditioned place preference test, PPI- Prepulse inhibition test, C/PPI- CPP test, followed by a PPI test, NAC- N-acetylcysteine and EUTH- Animal euthanized. CxRx- Refers to animal identification, i.e. C1R1 – Cage 1 Rat 1 etc. If identification code starts with a C (CxRx) – the animal was prenatally exposed to saline. Animal specific groups – SAL-OO-SAL- C1R1-C3R4, SAL-OO-NAC- C4R1-C6R4, SAL-EFV-SAL-C7R1-C9R4, SAL-EFV-NAC-C10R1-C11R1.

**Table 3:** Detailed outlay of experimental procedures from March – April 2019. The animals used (March-April) were prenatally exposed to either saline or lipopolysaccharide.

March 2019						
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
4	<b>5 48</b> C12R1- ND-CPP C12R2- ND-CPP C12R3- ND-CPP C12R4- ND-CPP  C13R1- ND-CPP C13R2- ND-CPP C13R3- ND-CPP  LC1R4- ND-CPP  LC2R1- ND-CPP  LC3R3- ND-CPP	<b>6 49</b> C12R1- E-CPP C12R2- E-CPP C12R3- E-CPP C12R4- E-CPP  C13R1- E-CPP C13R2- E-CPP C13R3- E-CPP  LC1R4- O-CPP  LC2R1- O-CPP  LC3R3- O-CPP	<b>7 50</b> C12R1- O-CPP C12R2- O-CPP C12R3- O-CPP C12R4- O-CPP  C13R1- O-CPP C13R2- O-CPP C13R3- O-CPP  LC1R4- O-CPP  LC2R1- O-CPP  LC3R3- O-CPP	<b>8 51</b> C12R1- E-CPP C12R2- E-CPP C12R3- E-CPP C12R4- E-CPP  C13R1- E-CPP C13R2- E-CPP C13R3- E-CPP  LC1R4- O-CPP  LC2R1- O-CPP  LC3R3- O-CPP	<b>9 52 48</b> C12R1- O-CPP C12R2- O-CPP C12R3- O-CPP C12R4- O-CPP  C13R1- O-CPP C13R2- O-CPP C13R3- O-CPP  LC1R2- ND-CPP LC1R3- ND-CPP LC1R4- O-CPP  LC2R1- O-CPP  LC2R4- ND-CPP  LC3R3- O-CPP	<b>10 53 49 48</b> C12R1- E-CPP C12R2- E-CPP C12R3- E-CPP C12R4- E-CPP  C13R1- E-CPP C13R2- E-CPP C13R3- E-CPP  LC1R2- O-CPP LC1R3- O-CPP LC1R4- O-CPP  LC2R1- O-CPP LC2R2- ND-CPP  LC2R4- O-CPP  LC3R1- ND-CPP LC3R2- ND-CPP LC3R3- O-CPP

Addendum B: Pre-natal and post-natal experimental protocols

	LC4R2- ND-CPP	LC4R2- O-CPP	LC4R2- O-CPP	LC4R2- O-CPP	LC4R2- O-CPP	LC4R1- ND-CPP LC4R2- O-CPP LC4R3- ND-CPP
					LC5R1- ND-CPP	LC5R1- O-CPP LC5R2- ND-CPP LC5R3- ND-CPP LC5R4- O-CPP
	LC6R3- ND-CPP	LC6R3- O-CPP	LC6R3- O-CPP	LC6R3-O-CPP	LC6R1- ND-CPP LC6R2- ND-CPP LC6R3- O-CPP LC6R4- ND-CPP	LC6R1- O-CPP LC6R2- O-CPP LC6R3-O-CPP LC6R4- O-CPP
	LC7R3- ND-CPP	LC7R3- E-CPP	LC7R3- O-CPP	LC7R3- E-CPP	LC7R2- ND-CPP LC7R3- O-CPP LC7R4- ND-CPP	LC7R1- ND-CPP LC7R2- E-CPP LC7R3- E-CPP LC7R4- E-CPP
	LC8R4- ND-CPP	LC8R4- E-CPP	LC8R4- O-CPP	LC8R4- E-CPP	LC8R3- ND-CPP LC8R4- O-CPP	LC8R1- ND-CPP LC8R2- ND-CPP LC8R3- E-CPP LC8R4- E-CPP
	LC9R2- ND-CPP LC9R3- ND-CPP	LC9R2- E-CPP LC9R3- E-CPP	LC9R2- O-CPP LC9R3- O-CPP	LC9R2- E-CPP LC9R3- E-CPP	LC9R1- ND-CPP LC9R2- O-CPP LC9R3- O-CPP LC9R4- ND-CPP	LC9R1- E-CPP LC9R2- E-CPP LC9R3- E-CPP LC9R4- E-CPP
	LC10R1- ND-CPP	LC10R1- E-CPP	LC10R1- O-CPP	LC10R1- E-CPP	LC10R1- O-CPP LC10R3- ND-CPP LC10R4- ND-CPP	LC10R1- E-CPP LC10R2- ND-CPP LC10R3- E-CPP LC10R4- E-CPP
					LC11R4- ND-CPP	LC11R2- ND-CPP LC11R3- ND-CPP LC11R4- E-CPP

Addendum B: Pre-natal and post-natal experimental protocols

	LC12R2- ND-CPP	LC12R2- E-CPP	LC12R2- O-CPP	LC12R2- E-CPP	LC12R2- O-CPP LC12R3- ND-CPP LC12R4- ND-CPP	LC12R1- ND-CPP LC12R2- E-CPP LC12R3- E-CPP LC12R4- E-CPP
<b>11 54 50 49 48</b> C12R1- O-CPP C12R2- O-CPP C12R3- O-CPP C12R4- O-CPP  C13R1- O-CPP C13R2- O-CPP C13R3- O-CPP  LC1R1- ND-CPP LC1R2- O-CPP LC1R3- O-CPP LC1R4- O-CPP  LC2R1- O-CPP LC2R2- O-CPP LC2R3- ND-CPP LC2R4- O-CPP  LC3R1- O-CPP LC3R2- O-CPP LC3R3- O-CPP LC3R4- ND-CPP  LC4R1- O-CPP LC4R2- O-CPP LC4R3- O-CPP LC4R4- ND-CPP  LC5R1- O-CPP LC5R2- O-CPP	<b>12 55 51 50 49</b> C12R1- ND-C/PPI C12R2- ND-C/PPI C12R3- ND-C/PPI C12R4- ND-C/PPI  C13R1- ND-C/PPI C13R2- ND-C/PPI C13R3- ND-C/PPI  LC1R1- O-CPP LC1R2- O-CPP LC1R3- O-CPP LC1R4- ND-C/PPI  LC2R1- ND-C/PPI LC2R2- O-CPP LC2R3- O-CPP LC2R4- O-CPP  LC3R1- O-CPP LC3R2- O-CPP LC3R3- ND-C/PPI LC3R4- O-CPP  LC4R1- O-CPP LC4R2- ND-C/PPI LC4R3- O-CPP LC4R4- O-CPP	<b>13 56 52 51 50</b> C12R1- NAC C12R2- NAC C12R3- NAC C12R4- NAC  C13R1- NAC C13R2- NAC C13R3- NAC  LC1R1- O-CPP LC1R2- O-CPP LC1R3- O-CPP LC1R4- SALINE  LC2R1- SALINE LC2R2- O-CPP LC2R3- O-CPP LC2R4- O-CPP  LC3R1- O-CPP LC3R2- O-CPP LC3R3- SALINE LC3R4- O-CPP  LC4R1- O-CPP LC4R2- NAC LC4R3- O-CPP LC4R4- O-CPP	<b>14 57 53 52 51</b> C12R1- NAC C12R2- NAC C12R3- NAC C12R4- NAC  C13R1- NAC C13R2- NAC C13R3- NAC  LC1R1- O-CPP LC1R2- O-CPP LC1R3- O-CPP LC1R4- SALINE  LC2R1- SALINE LC2R2- O-CPP LC2R3- O-CPP LC2R4- O-CPP  LC3R1- O-CPP LC3R2- O-CPP LC3R3- SALINE LC3R4- O-CPP  LC4R1- O-CPP LC4R2- NAC LC4R3- O-CPP LC4R4- O-CPP	<b>15 58 54 53 52</b> C12R1- NAC C12R2- NAC C12R3- NAC C12R4- NAC  C13R1- NAC C13R2- NAC C13R3- NAC  LC1R1- O-CPP LC1R2- O-CPP LC1R3- O-CPP LC1R4- SALINE  LC2R1- SALINE LC2R2- O-CPP LC2R3- O-CPP LC2R4- O-CPP  LC3R1- O-CPP LC3R2- O-CPP LC3R3- SALINE LC3R4- O-CPP  LC4R1- O-CPP LC4R2- NAC LC4R3- O-CPP LC4R4- O-CPP	<b>16 59 55 54 53</b> C12R1- NAC C12R2- NAC C12R3- NAC C12R4- NAC  C13R1- NAC C13R2- NAC C13R3- NAC  LC1R1- O-CPP LC1R2- ND-C/PPI LC1R3- ND-C/PPI LC1R4- SALINE  LC2R1- SALINE LC2R2- O-CPP LC2R3- O-CPP LC2R4- ND-C/PPI  LC3R1- O-CPP LC3R2- O-CPP LC3R3- SALINE LC3R4- O-CPP  LC4R1- O-CPP LC4R2- NAC LC4R3- O-CPP LC4R4- O-CPP  LC5R1- ND-C/PPI LC5R2- O-CPP	<b>17 60 56 55 54</b> C12R1- NAC C12R2- NAC C12R3- NAC C12R4- NAC  C13R1- NAC C13R2- NAC C13R3- NAC  LC1R1- O-CPP LC1R2- SALINE LC1R3- SALINE LC1R4- SALINE  LC2R1- SALINE LC2R2- ND-C/PPI LC2R3- O-CPP LC2R4- SALINE  LC3R1- ND-C/PPI LC3R2- ND-C/PPI LC3R3- SALINE LC3R4- O-CPP  LC4R1- ND-C/PPI LC4R2- NAC LC4R3- ND-C/PPI LC4R4- O-CPP  LC5R1- NAC LC5R2- ND-C/PPI

Addendum B: Pre-natal and post-natal experimental protocols

LC5R3- O-CPP LC5R4- O-CPP	LC5R3- O-CPP LC5R4- O-CPP	LC5R3- O-CPP LC5R4- O-CPP	LC5R3- O-CPP LC5R4- O-CPP	LC5R3- O-CPP LC5R4- O-CPP	LC5R3- O-CPP LC5R4- ND-C/PPI	LC5R3- ND-C/PPI LC5R4- NAC
LC6R1- O-CPP LC6R2- O-CPP LC6R3- O-CPP LC6R4- O-CPP	LC6R1- O-CPP LC6R2- O-CPP LC6R3- ND-C/PPI LC6R4- O-CPP	LC6R1- O-CPP LC6R2- O-CPP LC6R3- NAC LC6R4- O-CPP	LC6R1- O-CPP LC6R2- O-CPP LC6R3- NAC LC6R4- O-CPP	LC6R1- O-CPP LC6R2- O-CPP LC6R3- NAC LC6R4- O-CPP	LC6R1- ND-C/PPI LC6R2- ND-C/PPI LC6R3- NAC LC6R4- ND-C/PPI	LC6R1- NAC LC6R2- NAC LC6R3- NAC LC6R4- NAC
LC7R1- E-CPP LC7R2- O-CPP LC7R3- O-CPP LC7R4- O-CPP	LC7R1- O-CPP LC7R2- E-CPP LC7R3- ND-C/PPI LC7R4- E-CPP	LC7R1- E-CPP LC7R2- O-CPP LC7R3- SALINE LC7R4- O-CPP	LC7R1- O-CPP LC7R2- E-CPP LC7R3- SALINE LC7R4- E-CPP	LC7R1- E-CPP LC7R2- O-CPP LC7R3- SALINE LC7R4- O-CPP	LC7R1- O-CPP LC7R2- ND-C/PPI LC7R3- SALINE LC7R4- ND-C/PPI	LC7R1- ND-C/PPI LC7R2- SALINE LC7R3- SALINE LC7R4- SALINE
LC8R1- E-CPP LC8R2- E-CPP LC8R3- O-CPP LC8R4- O-CPP	LC8R1- O-CPP LC8R2- O-CPP LC8R3- E-CPP LC8R4- ND-C/PPI	LC8R1- E-CPP LC8R2- E-CPP LC8R3- O-CPP LC8R4- SALINE	LC8R1- O-CPP LC8R2- O-CPP LC8R3- E-CPP LC8R4- SALINE	LC8R1- E-CPP LC8R2- E-CPP LC8R3- O-CPP LC8R4- SALINE	LC8R1- O-CPP LC8R2- O-CPP LC8R3- ND-C/PPI LC8R4- SALINE	LC8R1- ND-C/PPI LC8R2- ND-C/PPI LC8R3- SALINE LC8R4- SALINE
LC9R1- O-CPP LC9R2- O-CPP LC9R3- O-CPP LC9R4- O-CPP	LC9R1- E-CPP LC9R2- ND-C/PPI LC9R3- ND-C/PPI LC9R4- E-CPP	LC9R1- O-CPP LC9R2- SALINE LC9R3- SALINE LC9R4- O-CPP	LC9R1- E-CPP LC9R2- SALINE LC9R3- SALINE LC9R4- E-CPP	LC9R1- O-CPP LC9R2- SALINE LC9R3- SALINE LC9R4- O-CPP	LC9R1- ND-C/PPI LC9R2- SALINE LC9R3- SALINE LC9R4- ND-C/PPI	LC9R1- SALINE LC9R2- SALINE LC9R3- SALINE LC9R4- SALINE
LC10R1- O-CPP LC10R2- E-CPP LC10R3- O-CPP LC10R4- O-CPP	LC10R1- ND-C/PPI LC10R2- O-CPP LC10R3- E-CPP LC10R4- E-CPP	LC10R1- NAC LC10R2- E-CPP LC10R3- O-CPP LC10R4- O-CPP	LC10R1- NAC LC10R2- O-CPP LC10R3- E-CPP LC10R4- E-CPP	LC10R1- NAC LC10R2- E-CPP LC10R3- O-CPP LC10R4- O-CPP	LC10R1- NAC LC10R2- O-CPP LC10R3- ND-C/PPI LC10R4- ND-C/PPI	LC10R1- NAC LC10R2- ND-C/PPI LC10R3- NAC LC10R4- NAC
LC11R1- ND-CPP LC11R2- E-CPP LC11R3- E-CPP LC11R4- O-CPP	LC11R1- E-CPP LC11R2- O-CPP LC11R3- O-CPP LC11R4- E-CPP	LC11R1- O-CPP LC11R2- E-CPP LC11R3- E-CPP LC11R4- O-CPP	LC11R1- E-CPP LC11R2- O-CPP LC11R3- O-CPP LC11R4- E-CPP	LC11R1- O-CPP LC11R2- E-CPP LC11R3- E-CPP LC11R4- O-CPP	LC11R1- E-CPP LC11R2- O-CPP LC11R3- O-CPP LC11R4- ND-C/PPI	LC11R1- O-CPP LC11R2- ND-C/PPI LC11R3- ND-C/PPI LC11R4- NAC
LC12R1- E-CPP LC12R2- O-CPP LC12R3- O-CPP LC12R4- O-CPP	LC12R1- O-CPP LC12R2- ND-C/PPI LC12R3- E-CPP LC12R4- E-CPP	LC12R1- E-CPP LC12R2- NAC LC12R3- O-CPP LC12R4- O-CPP	LC12R1- O-CPP LC12R2- NAC LC12R3- E-CPP LC12R4- E-CPP	LC12R1- E-CPP LC12R2- NAC LC12R3- O-CPP LC12R4- O-CPP	LC12R1- O-CPP LC12R2- NAC LC12R3- ND-C/PPI LC12R4- ND-C/PPI	LC12R1- ND-C/PPI LC12R2- NAC LC12R3- NAC LC12R4- NAC

Addendum B: Pre-natal and post-natal experimental protocols

18 61 57 56 55	19 62 58 57 56	20 63 59 58 57	21 64 60 59 58	22 65 61 60 59	23 66 62 61 60	24 67 63 62 61
C12R1- NAC	C12R1- NAC	C12R1- NAC	C12R1- NAC	C12R1- NAC	C12R1- NAC	C12R1- NAC
C12R2- NAC	C12R2- NAC	C12R2- NAC	C12R2- NAC	C12R2- NAC	C12R2- NAC	C12R2- NAC
C12R3- NAC	C12R3- NAC	C12R3- NAC	C12R3- NAC	C12R3- NAC	C12R3- NAC	C12R3- NAC
C12R4- NAC	C12R4- NAC	C12R4- NAC	C12R4- NAC	C12R4- NAC	C12R4- NAC	C12R4- NAC
C13R1- NAC	C13R1- NAC	C13R1- NAC	C13R1- NAC	C13R1- NAC	C13R1- NAC	C13R1- NAC
C13R2- NAC	C13R2- NAC	C13R2- NAC	C13R2- NAC	C13R2- NAC	C13R2- NAC	C13R2- NAC
C13R3- NAC	C13R3- NAC	C13R3- NAC	C13R3- NAC	C13R3- NAC	C13R3- NAC	C13R3- NAC
LC1R1- ND-C/PPI	LC1R1- SALINE					
LC1R2- SALINE	LC1R2- SALINE	LC1R2- SALINE	LC1R2- SALINE	LC1R2- SALINE	LC1R2- SALINE	LC1R2- SALINE
LC1R3- SALINE	LC1R3- SALINE	LC1R3- SALINE	LC1R3- SALINE	LC1R3- SALINE	LC1R3- SALINE	LC1R3- SALINE
LC1R4- SALINE	LC1R4- SALINE	LC1R4- SALINE	LC1R4- SALINE	LC1R4- SALINE	LC1R4- SALINE	LC1R4- SALINE
LC2R1- SALINE	LC2R1- SALINE	LC2R1- SALINE	LC2R1- SALINE	LC2R1- SALINE	LC2R1- SALINE	LC2R1- SALINE
LC2R2- SALINE	LC2R2- SALINE	LC2R2- SALINE	LC2R2- SALINE	LC2R2- SALINE	LC2R2- SALINE	LC2R2- SALINE
LC2R3- ND-C/PPI	LC2R3- SALINE					
LC2R4- SALINE	LC2R4- SALINE	LC2R4- SALINE	LC2R4- SALINE	LC2R4- SALINE	LC2R4- SALINE	LC2R4- SALINE
LC3R1- SALINE	LC3R1- SALINE	LC3R1- SALINE	LC3R1- SALINE	LC3R1- SALINE	LC3R1- SALINE	LC3R1- SALINE
LC3R2- SALINE	LC3R2- SALINE	LC3R2- SALINE	LC3R2- SALINE	LC3R2- SALINE	LC3R2- SALINE	LC3R2- SALINE
LC3R3- SALINE	LC3R3- SALINE	LC3R3- SALINE	LC3R3- SALINE	LC3R3- SALINE	LC3R3- SALINE	LC3R3- SALINE
LC3R4- ND-C/PPI	LC3R4- SALINE					
LC4R1- NAC	LC4R1- NAC	LC4R1- NAC	LC4R1- NAC	LC4R1- NAC	LC4R1- NAC	LC4R1- NAC
LC4R2- NAC	LC4R2- NAC	LC4R2- NAC	LC4R2- NAC	LC4R2- NAC	LC4R2- NAC	LC4R2- NAC
LC4R3- NAC	LC4R3- NAC	LC4R3- NAC	LC4R3- NAC	LC4R3- NAC	LC4R3- NAC	LC4R3- NAC
LC4R4- ND-C/PPI	LC4R4- NAC					
LC5R1- NAC	LC5R1- NAC	LC5R1- NAC	LC5R1- NAC	LC5R1- NAC	LC5R1- NAC	LC5R1- NAC
LC5R2- NAC	LC5R2- NAC	LC5R2- NAC	LC5R2- NAC	LC5R2- NAC	LC5R2- NAC	LC5R2- NAC
LC5R3- NAC	LC5R3- NAC	LC5R3- NAC	LC5R3- NAC	LC5R3- NAC	LC5R3- NAC	LC5R3- NAC
LC5R4- NAC	LC5R4- NAC	LC5R4- NAC	LC5R4- NAC	LC5R4- NAC	LC5R4- NAC	LC5R4- NAC
LC6R1- NAC	LC6R1- NAC	LC6R1- NAC	LC6R1- NAC	LC6R1- NAC	LC6R1- NAC	LC6R1- NAC
LC6R2- NAC	LC6R2- NAC	LC6R2- NAC	LC6R2- NAC	LC6R2- NAC	LC6R2- NAC	LC6R2- NAC

Addendum B: Pre-natal and post-natal experimental protocols

LC6R3-NAC LC6R4- NAC						
LC7R1- SALINE LC7R2- SALINE LC7R3- SALINE LC7R4- SALINE						
LC8R1- SALINE LC8R2- SALINE LC8R3- SALINE LC8R4- SALINE						
LC9R1- SALINE LC9R2- SALINE LC9R3- SALINE LC9R4- SALINE						
LC10R1- NAC LC10R2- NAC LC10R3- NAC LC10R4- NAC						
LC11R1-ND-C/PPI LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC
LC12R1- NAC LC12R2- NAC LC12R3- NAC LC12R4- NAC						

Addendum B: Pre-natal and post-natal experimental protocols

25 68 64 63 62 C12R1- NAC C12R2- NAC C12R3- NAC C12R4- NAC  C13R1- NAC C13R2- NAC C13R3- NAC  LC1R1- SALINE LC1R2- SALINE LC1R3- SALINE LC1R4- SALINE  LC2R1- SALINE LC2R2- SALINE LC2R3- SALINE LC2R4- SALINE  LC3R1- SALINE LC3R2- SALINE LC3R3- SALINE LC3R4- SALINE  LC4R1- NAC LC4R2- NAC LC4R3- NAC LC4R4- NAC  LC5R1- NAC LC5R2- NAC LC5R3- NAC LC5R4- NAC  LC6R1- NAC LC6R2- NAC	26 69 65 64 63 C12R1- NAC C12R2- NAC C12R3- NAC C12R4- NAC  C13R1- NAC C13R2- NAC C13R3- NAC  LC1R1- SALINE LC1R2- SALINE LC1R3- SALINE LC1R4- SALINE  LC2R1- SALINE LC2R2- SALINE LC2R3- SALINE LC2R4- SALINE  LC3R1- SALINE LC3R2- SALINE LC3R3- SALINE LC3R4- SALINE  LC4R1- NAC LC4R2- NAC LC4R3- NAC LC4R4- NAC  LC5R1- NAC LC5R2- NAC LC5R3- NAC LC5R4- NAC  LC6R1- NAC LC6R2- NAC	27 70 66 65 64 C12R1- E-CPP C12R2- E-CPP C12R3- E-CPP C12R4- E-CPP  C13R1- E-CPP C13R2- E-CPP C13R3- E-CPP  LC1R1- SALINE LC1R2- SALINE LC1R3- SALINE LC1R4- O-CPP  LC2R1- O-CPP LC2R2- SALINE LC2R3- SALINE LC2R4- SALINE  LC3R1- SALINE LC3R2- SALINE LC3R3- O-CPP LC3R4- SALINE  LC4R1- NAC LC4R2- O-CPP LC4R3- NAC LC4R4- NAC  LC5R1- NAC LC5R2- NAC LC5R3- NAC LC5R4- NAC  LC6R1- NAC LC6R2- NAC	28 71 67 66 65 C12R1- O-CPP C12R2- O-CPP C12R3- O-CPP C12R4- O-CPP  C13R1- O-CPP C13R2- O-CPP C13R3- O-CPP  LC1R1- SALINE LC1R2- SALINE LC1R3- SALINE LC1R4- O-CPP  LC2R1- O-CPP LC2R2- SALINE LC2R3- SALINE LC2R4- SALINE  LC3R1- SALINE LC3R2- SALINE LC3R3- O-CPP LC3R4- SALINE  LC4R1- NAC LC4R2- O-CPP LC4R3- NAC LC4R4- NAC  LC5R1- NAC LC5R2- NAC LC5R3- NAC LC5R4- NAC  LC6R1- NAC LC6R2- NAC	29 72 68 67 66 C12R1- ND-C/PPI C12R2- ND-C/PPI C12R3- ND-C/PPI C12R4- ND-C/PPI  C13R1- ND-C/PPI C13R2- ND-C/PPI C13R3- ND-C/PPI  LC1R1- SALINE LC1R2- SALINE LC1R3- SALINE LC1R4- ND-C/PPI  LC2R1- ND-C/PPI LC2R2- SALINE LC2R3- SALINE LC2R4- SALINE  LC3R1- SALINE LC3R2- SALINE LC3R3- ND-C/PPI LC3R4- SALINE  LC4R1- NAC LC4R2- ND-C/PPI LC4R3- NAC LC4R4- NAC  LC5R1- NAC LC5R2- NAC LC5R3- NAC LC5R4- NAC  LC6R1- NAC LC6R2- NAC	30 73 69 68 67 C12R1- EUTH C12R2- EUTH C12R3- EUTH C12R4- EUTH  C13R1- EUTH C13R2- EUTH C13R3- EUTH  LC1R1- SALINE LC1R2- SALINE LC1R3- SALINE LC1R4- EUTH  LC2R1- EUTH LC2R2- SALINE LC2R3- SALINE LC2R4- SALINE  LC3R1- SALINE LC3R2- SALINE LC3R3- EUTH LC3R4- SALINE  LC4R1- NAC LC4R2- EUTH LC4R3- NAC LC4R4- NAC  LC5R1- NAC LC5R2- NAC LC5R3- NAC LC5R4- NAC  LC6R1- NAC LC6R2- NAC	31 70 69 68 LC1R1- SALINE LC1R2- O-CPP LC1R3- O-CPP  LC2R2- SALINE LC2R3- SALINE LC2R4- O-CPP  LC3R1- SALINE LC3R2- SALINE LC3R4- SALINE  LC4R1- NAC LC4R3- NAC LC4R4- NAC  LC5R1- O-CPP LC5R2- NAC LC5R3- NAC LC5R4- O-CPP  LC6R1- O-CPP LC6R2- O-CPP
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Addendum B: Pre-natal and post-natal experimental protocols

LC6R3-NAC LC6R4- NAC	LC6R3-NAC LC6R4- NAC	LC6R3-O-CPP LC6R4- NAC	LC6R3-O-CPP LC6R4- NAC	LC6R3-ND-C/PPI LC6R4- NAC	LC6R3-EUTH LC6R4- NAC	LC6R4- O-CPP
LC7R1- SALINE LC7R2- SALINE LC7R3- SALINE LC7R4- SALINE	LC7R1- SALINE LC7R2- SALINE LC7R3- SALINE LC7R4- SALINE	LC7R1- SALINE LC7R2- SALINE LC7R3- E-CPP LC7R4- SALINE	LC7R1- SALINE LC7R2- SALINE LC7R3- O-CPP LC7R4- SALINE	LC7R1- SALINE LC7R2- SALINE LC7R3- ND-C/PPI LC7R4- SALINE	LC7R1- SALINE LC7R2- SALINE LC7R4- SALINE LC7R3- EUTH	LC7R1- SALINE LC7R2- E-CPP LC7R4- E-CPP
LC8R1- SALINE LC8R2- SALINE LC8R3- SALINE LC8R4- SALINE	LC8R1- SALINE LC8R2- SALINE LC8R3- SALINE LC8R4- SALINE	LC8R1- SALINE LC8R2- SALINE LC8R3- SALINE LC8R4- E-CPP	LC8R1- SALINE LC8R2- SALINE LC8R3- SALINE LC8R4- O-CPP	LC8R1- SALINE LC8R2- SALINE LC8R3- SALINE LC8R4- ND-C/PPI	LC8R1- SALINE LC8R2- SALINE LC8R3- SALINE LC8R4- EUTH	LC8R1- SALINE LC8R2- SALINE LC8R3- E-CPP
LC9R1- SALINE LC9R2- SALINE LC9R3- SALINE LC9R4- SALINE	LC9R1- SALINE LC9R2- SALINE LC9R3- SALINE LC9R4- SALINE	LC9R1- SALINE LC9R2- E-CPP LC9R3- E-CPP LC9R4- SALINE	LC9R1- SALINE LC9R2- O-CPP LC9R3- O-CPP LC9R4- SALINE	LC9R1- SALINE LC9R2- ND-C/PPI LC9R3- ND-C/PPI LC9R4- SALINE	LC9R1- SALINE LC9R2- EUTH LC9R3- EUTH LC9R4- SALINE	LC9R1- E-CPP  LC9R4- E-CPP
LC10R1- NAC LC10R2- NAC LC10R3- NAC LC10R4- NAC	LC10R1- NAC LC10R2- NAC LC10R3- NAC LC10R4- NAC	LC10R1- E-CPP LC10R2- NAC LC10R3- NAC LC10R4- NAC	LC10R1- O-CPP LC10R2- NAC LC10R3- NAC LC10R4- NAC	LC10R1- ND-C/PPI LC10R2- NAC LC10R3- NAC LC10R4- NAC	LC10R1- EUTH LC10R2- NAC LC10R3- NAC LC10R4- NAC	LC10R2- NAC LC10R3- E-CPP LC10R4- E-CPP
LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- NAC	LC11R1-NAC LC11R2- NAC LC11R3- NAC LC11R4- E-CPP
LC12R1- NAC LC12R2- NAC LC12R3- NAC LC12R4- NAC	LC12R1- NAC LC12R2- NAC LC12R3- NAC LC12R4- NAC	LC12R1- NAC LC12R2- E-CPP LC12R3- NAC LC12R4- NAC	LC12R1- NAC LC12R2- O-CPP LC12R3- NAC LC12R4- NAC	LC12R1- NAC LC12R2- ND-C/PPI LC12R3- NAC LC12R4- NAC	LC12R1- NAC LC12R2- EUTH LC12R3- NAC LC12R4- NAC	LC12R1- NAC  LC12R3- E-CPP LC12R4- E-CPP
April 2019						
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1 71 70 69 LC1R1- SALINE LC1R2- O-CPP	2 72 71 70 LC1R1- O-CPP LC1R2- ND-C/PPI	3 73 72 71 LC1R1- O-CPP LC1R2- EUTH	4 73 72 LC1R1- ND-C/PPI	5 73 LC1R1- EUTH	6	7

Addendum B: Pre-natal and post-natal experimental protocols

LC1R3- O-CPP	LC1R3- ND-C/PPI	LC1R3- EUTH			
LC2R2- O-CPP	LC2R2- O-CPP	LC2R2- ND-C/PPI	LC2R2- EUTH		
LC2R3- SALINE	LC2R3- O-CPP	LC2R3- O-CPP	LC2R3- ND-C/PPI	LC2R3- EUTH	
LC2R4- O-CPP	LC2R4- ND-C/PPI	LC2R4- EUTH			
LC3R1- O-CPP	LC3R1- O-CPP	LC3R1- ND-C/PPI	LC3R1- EUTH		
LC3R2- O-CPP	LC3R2- O-CPP	LC3R2- ND-C/PPI	LC3R2- EUTH		
LC3R4- SALINE	LC3R4- O-CPP	LC3R4- O-CPP	LC3R4- ND-C/PPI	LC3R4- EUTH	
LC4R1- O-CPP	LC4R1- O-CPP	LC4R1- ND-C/PPI	LC4R1- EUTH		
LC4R3- O-CPP	LC4R3- O-CPP	LC4R3- ND-C/PPI	LC4R3- EUTH		
LC4R4- NAC	LC4R4- O-CPP	LC4R4- O-CPP	LC4R4- ND-C/PPI	LC4R4- EUTH	
LC5R1- O-CPP	LC5R1- ND-C/PPI	LC5R1- EUTH			
LC5R2- O-CPP	LC5R2- O-CPP	LC5R2- ND-C/PPI	LC5R2- EUTH		
LC5R3- O-CPP	LC5R3- O-CPP	LC5R3- ND-C/PPI	LC5R3- EUTH		
LC5R4- O-CPP	LC5R4- ND-C/PPI	LC5R4- EUTH			
LC6R1- O-CPP	LC6R1- ND-C/PPI	LC6R1- EUTH			
LC6R2- O-CPP	LC6R2- ND-C/PPI	LC6R2- EUTH			
LC6R4- O-CPP	LC6R4- ND-C/PPI	LC6R4- EUTH			
LC7R1- E-CPP	LC7R1- O-CPP	LC7R1- ND-C/PPI	LC7R1- EUTH		
LC7R2- O-CPP	LC7R2- ND-C/PPI	LC7R2- EUTH			
LC7R4- O-CPP	LC7R4- ND-C/PPI	LC7R4- EUTH			
LC8R1- E-CPP	LC8R1- O-CPP	LC8R1- ND-C/PPI	LC8R1- EUTH		
LC8R2- E-CPP	LC8R2- O-CPP	LC8R2- ND-C/PPI	LC8R2- EUTH		
LC8R3- O-CPP	LC8R3- ND-C/PPI	LC8R3- EUTH			

Addendum B: Pre-natal and post-natal experimental protocols

<b>LC9R1- O-CPP</b>	<b>LC9R1- ND-C/PPI</b>	<b>LC9R1- EUTH</b>			
<b>LC9R4- O-CPP</b>	<b>LC9R4- ND-C/PPI</b>	<b>LC9R4- EUTH</b>			
<b>LC10R2- E-CPP</b> <b>LC10R3- O-CPP</b> <b>LC10R4- O-CPP</b>	<b>LC10R2- O-CPP</b> <b>LC10R3- ND-C/PPI</b> <b>LC10R4- ND-C/PPI</b>	<b>LC10R2- ND-C/PPI</b> <b>LC10R3- EUTH</b> <b>LC10R4- EUTH</b>	<b>LC10R2- EUTH</b>		
<b>LC11R1- NAC</b> <b>LC11R2- E-CPP</b> <b>LC11R3- E-CPP</b> <b>LC11R4- O-CPP</b>	<b>LC11R1-E-CPP</b> <b>LC11R2- O-CPP</b> <b>LC11R3- O-CPP</b> <b>LC11R4- ND-C/PPI</b>	<b>LC11R1-O-CPP</b> <b>LC11R2- ND-C/PPI</b> <b>LC11R3- ND-C/PPI</b> <b>LC11R4- EUTH</b>	<b>LC11R1-ND-C/PPI</b> <b>LC11R2- EUTH</b> <b>LC11R3- EUTH</b>	<b>LC11R1- EUTH</b>	
<b>LC12R1- E-CPP</b>	<b>LC12R1- O-CPP</b>	<b>LC12R1- ND-C/PPI</b>	<b>LC12R1- EUTH</b>		
<b>LC12R3- O-CPP</b> <b>LC12R4- O-CPP</b>	<b>LC12R3- ND-C/PPI</b> <b>LC12R4- ND-C/PPI</b>	<b>LC12R3- EUTH</b> <b>LC12R4- EUTH</b>			

Dates of birth: **16/01/2019**, **20/01/2019**, **21/01/2019**, **22/01/2019**. Abbreviations: E- Efavirenz, O- Olive oil, ND- No drug administered, CPP- Conditioned place preference test, PPI- Prepulse inhibition test, C/PPI- CPP test, followed by a PPI test, NAC- N-acetylcysteine and EUTH- Animal euthanized. CxRx- Refers to animal identification, i.e. C1R1 – Cage 1 Rat 1 etc. If identification code starts with a C (CxRx) – the animal was prenatally exposed to saline. If identification code starts with a L (LCxRx) – the animal was prenatally exposed to lipopolysaccharide. Animal specific groups – SAL-EFV-NAC- C12R1-C13R3, LPS-OO-SAL- LC1R1-LC3R4, LPS-OO-NAC- LC4R1-LC6R4, LPS-EFV-SAL- LC7R1-LC9R4, LPS-EFV-NAC- C10R1-C12R4.

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## **ADDENDUM C**

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### **PERIPHERAL- AND NEUROCHEMICAL ANALYSES**

**Addendum C serves as a comprehensive explanation of the methodologies followed for all peripheral (plasma)- and neurochemical (cerebellum, frontal cortex (FC) and striatum) analyses performed throughout this study.**

Peripheral analyses performed: ‘

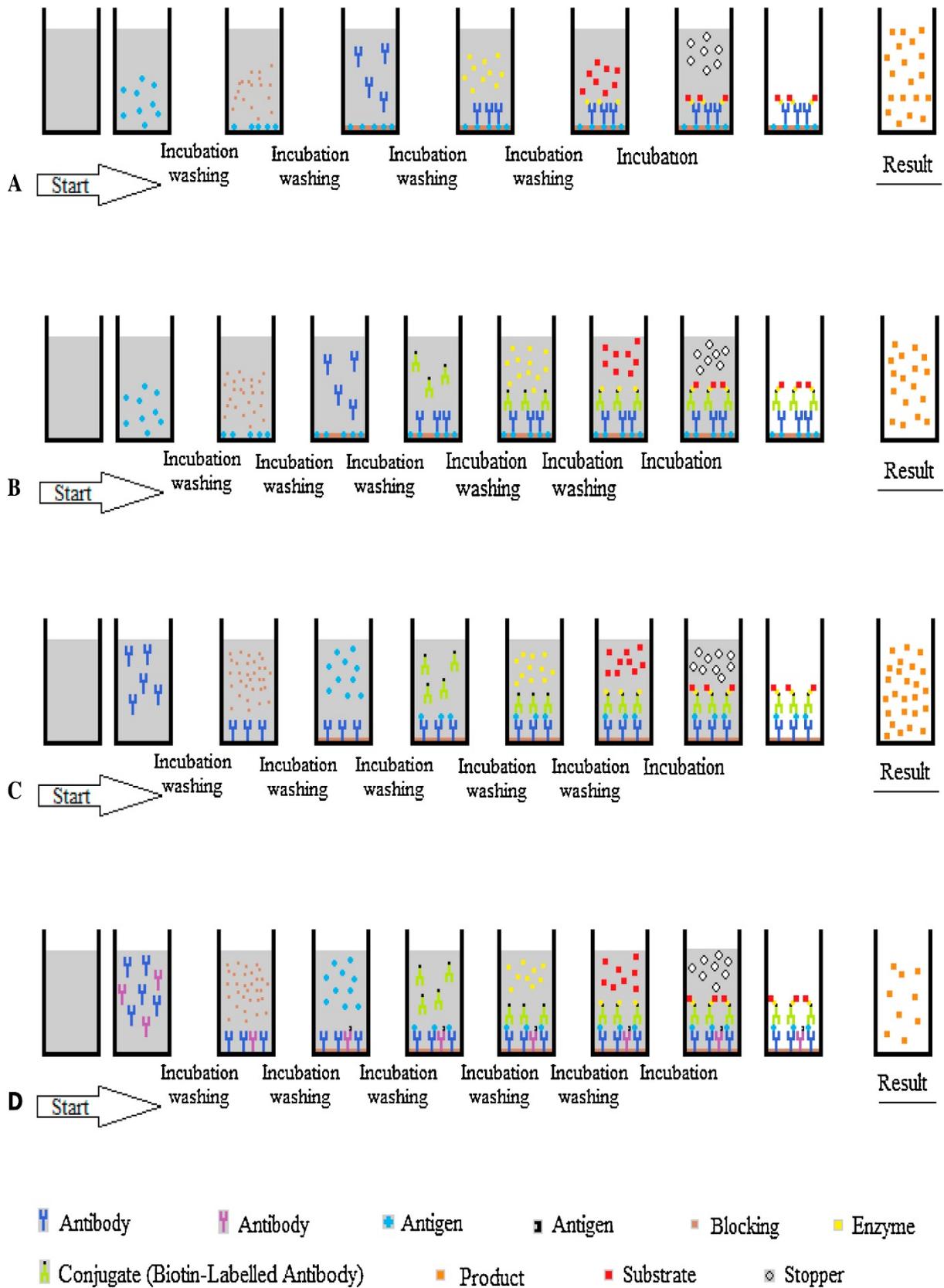
1. Corticosterone (CORT)
2. Glutathione (GSH)

Neurochemical analyses performed:

3. c-Fos
4. Dopamine transporter (DAT)
5. Phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B)

For all peripheral and neurochemical analyses, sandwich or competitive enzyme linked immunosorbent assay (ELISA) kits from different manufacturers (Elabscience®, MyBioSource & Bioassay Technology Laboratory) were used. The ELISA method was established in 1971 (Engvall and Perlmann, 1971) as an alternative to radioimmunoassay methods (Van Weemen and Schuurs, 1971). Since the development of this method, ELISA kits have been used globally due to distinct advantages such as; cost-effectiveness, reagents with long lifespans, absence of radiation exposure risks and the ability to analyse multiple samples within a very short time period (Aydin, 2015).

Enzymatic immunoassay methods can be categorized under two general classifications i.e. homogenous and heterogenous (O’Kennedy et al., 1990). The homogenous method is employed to detect a substance in small quantities, such as a therapeutic drug (O’Kennedy et al., 1990). The method is expensive and has a very low sensitivity, with only one advantage which is being easy to use (Aydin, 2015). The heterogenous methods are used more frequently (O’Kennedy et al., 1990) due to superior sensitivity (Aydin, 2015). ELISA is classified as a heterogenous immunoassay technique implemented to detect soluble antigens and specific antibodies (Aydin, 2015). Importantly, characteristics and structures of target substances differ significantly from one another, and therefor different ELISA types have been created to increase the specificity of measurement (O’Kennedy et al., 1990). For a schematic description on ELISA methods see figure 1.



**Figure 1:** A schematic representation of enzyme linked immunosorbent assay (ELISA) methods (Aydin, 2015). A – Direct method, B- Indirect method, C- Sandwich method and D – Competitive method.

### *Direct ELISA method*

The direct ELISA method was developed in 1971 (Engvall and Perlmann, 1971) and initiated all other ELISA types (Aydin, 2015). This method is appropriate for measuring the amount of high molecular-weight antigens (Aydin, 2015). Despite this method being rather quick to perform, it is not very sensitive and may produce a false positive result (Aydin, 2015). The method works as follows (Figure 1: Aydin, 2015);

1. The plate surface is coated directly with the antigen or antibody.
2. An enzyme tagged antigen or antibody allows the measurement.
3. Incubation, followed by washing will remove unbound antibodies or antigens from the medium.
4. The appropriate substrate is then added to the medium to bring about a signal through colouration.
5. The amount of antibodies or antigens is determined by measuring the signal.

### *Indirect ELISA method*

The highly-sensitive indirect ELISA method was set out by (Lindström and Wager, 1978). Unlike the direct method, the separation and determination of the antigen measured is not via the primary antibody, but via a secondary antibody placed within the medium (Aydin, 2015). The method works as follows (Figure 1: Aydin, 2015);

1. The wells are coated with antigens. Diseased serum is then added to these wells and the plates are then incubated.
2. During incubation – An antigen-antibody complex is formed. This is via the antibodies that formed against the antigens within the serum plaque.
3. A secondary antibody is added to make the antigen-antibody complex visible. This secondary antibody recognizes the antibody (tagged with an enzyme previously added) within the serum.
4. A substrate of the enzyme is added to the medium. This will produce a colour and a concentration can now be determined.

### *Sandwich ELISA method (antibody screening)*

The sandwich ELISA method was described by (Kato et al., 1977). This method is up to five times more sensitive compared to all other ELISA's. The method works as follows (Figure 1: Aydin, 2015);

1. The wells in the plate are coated with a capture antibody and blocked.
2. The sample is added, thereafter the plate is incubated and washed. The washing step is to ensure the removal of unbound antigens.

3. Antibodies (tagged with antigen specific enzyme) are added and incubated and washed.
4. To indicate enzyme activity, an enzyme substrate is added to the medium and colouration will take place.
5. Colouration indicate a positive result, as the lack thereof indicated a negative result or lack of enzymes.

*Competitive ELISA method (antigen/antibody screening)*

The competitive method was also developed in 1977, by the same team of researchers that produced the sandwich ELISA method (Kato et al., 1977). The method works as follows (Figure 1) (Aydin, 2015);

1. The plate surface is coated with antibody-specific antigen or antigen-specific antibody.
2. The enzyme-tagged antibody or antigen along with the sample are added into the wells.
3. The untagged antigen or antibody (patient antigen or antibody) and tagged antigen or antibody molecules compete with one another to bind to the antigen or antibody within the wells.
4. The wells are then washed. Thereafter, an enzyme substrate is added and colouration will take place. The colouration will enable the researcher to quantify the concentration.

## **C.1 CORT**

### **C.1.1 Introduction**

CORT is the primary stress hormone in rodents and serves as an excellent biomarker to characterize the stress response within experimental animals (Bekhbat et al., 2018). CORT concentrations within rat plasma have been determined in previous pre-clinical studies using ELISA kits (Wang et al., 2018, Bekhbat et al., 2018). In vitro quantitative determination of rat plasma CORT in this study was analysed by using the Rat CORT (corticosterone) ELISA kit catalog No: E-EL-R0269 from Elabscience (United States of America; USA).

#### Certificate of analysis

- Production No.: E-EL-R0269
- Lot No.: 1FF14R7VKT
- Date: 2019-02-13

#### Specification

- Sensitivity: 1.88 ng/ml
- Detection Range: 3.13-200 ng/ml
- Specificity: CORT in given sample. No significant interference or cross-reactivity between rat CORT and analogues was found.
- Repeatability: Coefficient of variation is <10%.

#### Principle of test

Elabscience's ELISA kit depended on a competitive-ELISA principle. Rat CORT was pre-coated on the provided microtiter plates. In the case of a reaction, the rat CORT (present in the sample or standard) competed with the rat CORT on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to rat CORT. Any unbound sample/standard and excess conjugate was washed away from the plates. Avidin conjugated to Horseradish Peroxidase (HRP) was added to the microplate wells and incubated afterwards. Next, 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to the wells. To terminate the enzyme-substrate reaction, a stop solution was added and a change in colour occurred. The colour change was spectrophotometrically measured at a wavelength of 450 nm  $\pm$  2 nm. To determine the CORT concentration in the given samples, the optical density (OD) of the given samples were compared to those of the standard curve.

### **C.1.2 Materials**

#### Provided materials:

- Manual
- Certificate of Analysis
- Micro ELISA plate (8 wells x 12 strips)
- Plate sealer (5 pieces)
- Reference Standard (2 vials)
- Concentrated Biotinylated Detection Antibody (100x – 1 vial 120 µl)
- Concentrated HRP Conjugate (100x – 1 vial 120 µl)
- Reference standard & Sample diluent (1 vial 20 ml)
- Biotinylated Detection Antibody Diluent (1 vial 14 ml)
- HRP Conjugate Diluent (1 vial 14 ml)
- Concentrated Wash buffer (25x – 1 vial 30 ml)
- Substrate reagent (1 vial 10 ml)
- Stop solution (1 vial 10 ml)

#### Other materials required:

- Microplate reader with 450 nm wavelength filter BioTek FL600  
Microplate Fluorescence reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA)
- Incubator (37°C) SHAKER DTS-4
- High-precision transfer pipette
- Eppendorf (EP) tubes and disposable pipette tips
- Distilled water
- Absorbent paper
- Loading slot for wash buffer BioTek

### **C.1.3 Sample preparation**

1. After the animals were euthanized, trunk blood was collected in pre-chilled Vacuette® tubes containing a K<sub>2</sub> Ethylenediaminetetraacetic acid (EDTA) solution as anti-coagulant.
2. The samples were centrifuged – 15 minutes at 1000xg (2-8°C) within 30 minutes after collection.
3. The supernatant was collected for assay.
4. Samples were stored at -80°C (≤ 3 months).

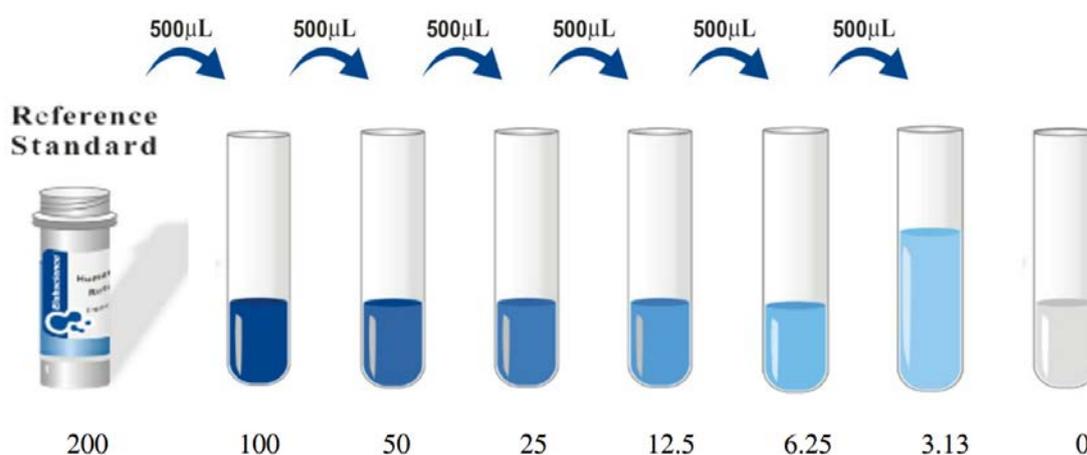
### C.1.4 Reagent preparation

1. Before the procedure started, all reagents were brought to room temperature (18-25°C).
2. The microplate reader was pre-heated.
3. The wash buffer was prepared

To prepare 750 ml wash buffer – 30 ml of the concentrated wash buffer was diluted with 720 ml distilled water.

4. The standard working solution was prepared as follows:

The standard was centrifuged– 10000xg (60 seconds). 1 ml of reference standard and sample diluent (left to stand for 10 minutes) was added and overturned gently a few times. Once fully dissolved, the solution was carefully mixed with a pipette. This produced a 200 ng/ml working solution. Serial dilutions were prepared (200, 100, 50, 25, 12.5, 6.25, 3.13, 0 ng/ml were recommended). A visual demonstration of the dilution is presented in figure 2. 7 EP tubes were prepared and 500 µl of reference standard and 500 µl sample diluent were added to each one. 500 µl of the 200 ng/ml working solution was pipetted to the first EP tube and mix thoroughly to produce a 100 ng/ml working solution. This process was continued by pipetting 500 µl from the former EP tube to the next. The last EP tube served as a blank.



**Figure 2:** Dilution method for working solution (figure was taken from the manual provided by the manufacturer).

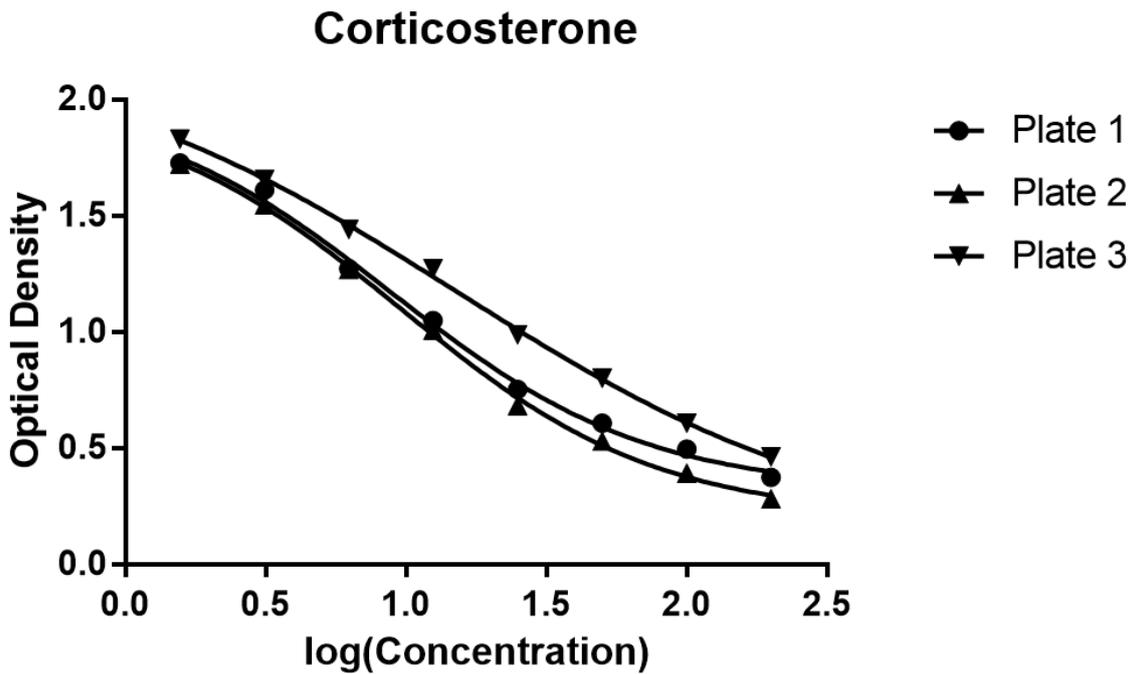
5. The Biotinylated Detection Antibody working solution was prepared as follows:  
The exact amount was calculated before the experiment (50 µl/well). The stock tube was centrifuged. 1x working solution with Biotinylated Detection Antibody Diluent was diluted with 100x Concentrated Biotinylated Detection Antibody.
6. The concentrated HRP Conjugate working solution was prepared  
The exact amount was calculated before the experiment (100 µl/well). 1x working solution with Concentrated HRP Conjugate Diluent was diluted with 100x Concentrated HRP Conjugate.

### **C.1.5 Assay procedure**

1. The standard working solution (50 µl) was added in the first two columns. All concentrations of the solution were added in duplicate.
2. A sample (50 µl) was added to each individual well, this also occurred in duplicate.
3. The biotinylated Detection Ab (50 µl) was added immediately to each individual well.
4. The plates were covered with one of the provided sealers and incubate (45 minutes - 37°C). SHAKER DTS-4 were used as an incubator.
5. The plates were aspirated and washed three times. This was done by adding the buffer (350 µl) to each well and allowing them to soak for 2 minutes before decanting the buffer. After this step, the plates were patted dry against absorbent paper. The plates were washed automatically by a microplate washer (BioTek) in the laboratory.
6. Addition of HRP Conjugate (100 µl) to each individual well.
7. The plates were covered with one of the provided sealers and incubated (30 minutes - 37°C).
8. The plates were aspirated and washed five times, as described in step 5.
9. The substrate reagent (90 µl) was added to each individual well.
10. The plates were covered with one of the provided sealers and incubated (15 minutes - 37°C).
11. The stop solution (50 µl) was added to each individual well.
12. The OD value (450 nm) was determined immediately. This was done by using a microplate reader.
13. The results were calculated.

### D.1.6 Calculation of results

The average of each sample and standard were calculated, since assays were performed in duplicate. Computer software capable of plotting a four-parameter logistic curve was used. The x-axis represented the standard concentration and the y-axis the OD values. See figure 3 for the logistic curve produced by the rat CORT concentration in this experiment for three plates. Three plates were needed as 96 samples had to be analysed in duplicate. Please see figure 4 for a detailed plate layout for all three plates used.



**Figure 3:** Standard logistic curve for rat corticosterone (CORT) measured in plasma.

#### Rat sample allocation:

- 1 – 12 = Saline (SAL) – Olive oil (OO) – SAL group
- 13 – 24 = SAL – OO – N-acetylcysteine (NAC) group
- 25 – 36 = SAL – efavirenz (EFV) – SAL group
- 37 – 48 = SAL – EFV – NAC group
- 49 – 60 = Lipopolysaccharide (LPS) – OO – SAL group
- 61 – 72 = LPS – OO – NAC group
- 73 – 84 = LPS – EFV – SAL group
- 85 – 96 = LPS – EFV – NAC group

**Plate 1** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
A	0	0	1	1	9	9	17	17	25	25	33	33
B	3,13	3,13	2	2	10	10	18	18	26	26	34	34
C	6,25	6,25	3	3	11	11	19	19	27	27	35	35
D	12,5	12,5	4	4	12	12	20	20	28	28	36	36
E	25	25	5	5	13	13	21	21	29	29	37	37
F	50	50	6	6	14	14	22	22	30	30	38	38
G	100	100	7	7	15	15	23	23	31	31	39	39
H	200	200	8	8	16	16	24	24	32	32	40	40

**Plate 2** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
A	0	0	41	41	49	49	57	57	65	65	73	73
B	3,13	3,13	42	42	50	50	58	58	66	66	74	74
C	6,25	6,25	43	43	51	51	59	59	67	67	75	75
D	12,5	12,5	44	44	52	52	60	60	68	68	76	76
E	25	25	45	45	53	53	61	61	69	69	77	77
F	50	50	46	46	54	54	62	62	70	70	78	78
G	100	100	47	47	55	55	63	63	71	71	79	79
H	200	200	48	48	56	56	64	64	72	72	80	80

**Plate 3** – Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
A	0	0	81	81	89	89						
B	3,13	3,13	82	82	90	90						
C	6,25	6,25	83	83	91	91						
D	12,5	12,5	84	84	92	92						
E	25	25	85	85	93	93						
F	50	50	86	86	94	94						
G	100	100	87	87	95	95						
H	200	200	88	88	96	96						

**Figure 4:** Plate 1- 3 layout for rat corticosterone (CORT) measured in plasma.

## **C.2 GSH**

### **C.2.1 Introduction**

GSH is a tripeptide (Rosa et al., 2014) consisting of the amino acids cysteine, glycine and glutamate (Zhang et al., 2008). Measurement of GSH in the plasma is an indicator of whole organism oxidative status (Schafer and Buettner, 2001, Jones, 2006). In vitro quantitative determination of GSH in this study was analysed by using the GSH ELISA kit catalog No: E-EL-0026 from Elabscience (USA).

#### Certificate of analysis

- Production No.: E-EL-0026
- Lot No.: ZELACZ2J2Z
- Date: 2019-02-13

#### Specification

- Sensitivity: 0.94 µg/ml
- Detection Range: 1.56-100 µg/ml
- Specificity: GSH in given sample. No significant interference or cross-reactivity between GSH and analogues was found.
- Repeatability: Coefficient of variation is <10%.

#### Principle of test

Elabscience's ELISA kit depended on a competitive-ELISA principle. GSH was pre-coated on the provided microtiter plates. In the case of a reaction, the GSH (present in the sample or standard) competed with the GSH on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to GSH. Any unbound sample/standard and excess conjugate were washed away from the plates. Avidin conjugated to HRP were then added to the microplate wells and incubated afterwards. Next, TMB substrate solution was added to the wells. To terminate the enzyme-substrate reaction a stop solution was added and a change in colour occurred. The colour change was spectrophotometrically measured at a wavelength of 450 nm ± 2 nm. The GSH concentration in the given samples was determined by comparing the OD of the given samples to the standard curve.

### **C.2.2 Materials**

#### Provided materials:

- Manual

- Certificate of Analysis
- Micro ELISA plate (8 wells x 12 strips)
- Plate sealer (5 pieces)
- Reference Standard (2 vials)
- Concentrated Biotinylated Detection Antibody (100x – 1 vial 120 µl)
- Concentrated HRP Conjugate (100x – 1 vial 120 µl)
- Reference standard & Sample diluent (1 vial 20 ml)
- Biotinylated Detection Antibody Diluent (1 vial 14 ml)
- HRP Conjugate Diluent (1 vial 14 ml)
- Concentrated Wash buffer (25x – 1 vial 30 ml)
- Substrate reagent (1 vial 10 ml)
- Stop solution (1 vial 10 ml)

Other materials required:

- Microplate reader with 450nm wavelength filter BioTek FL600  
Microplate Fluorescence reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA)
- Incubator (37°C) SHAKER DTS-4
- High-precision transfer pipette
- EP tubes and disposable pipette tips
- Distilled water
- Absorbent paper
- Loading slot for wash buffer BioTek

### **C.2.3 Sample preparation**

1. After the animals were euthanized, trunk blood was collected in pre-chilled Vacuette® tubes containing a K<sub>2</sub>EDTA solution as anti-coagulant.
2. The samples were centrifuged – 15 minutes at 1000xg (2-8°C) within 30 minutes after collection.
3. The supernatant was collected for assay.
4. Samples were stored at -80°C (≤ 3 months).

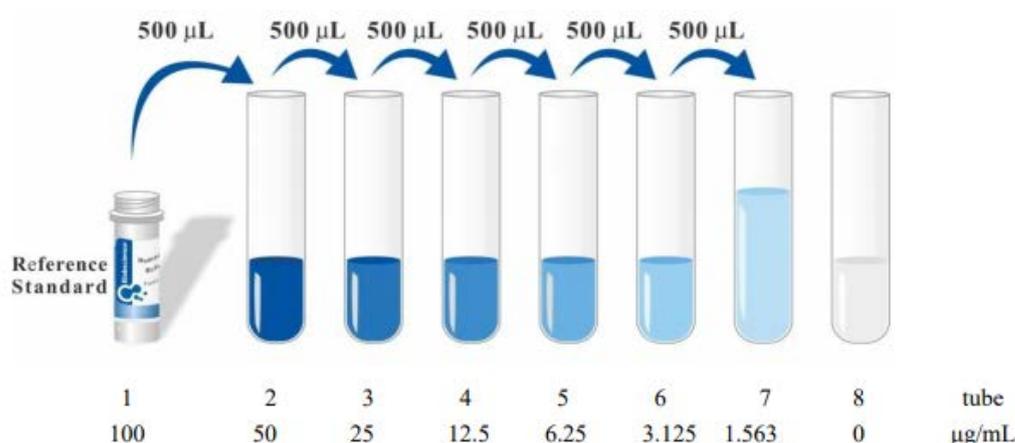
### **C.2.4 Reagent preparation**

1. Before the procedure started, all reagents were brought to room temperature (18-25°C).
2. The microplate reader was pre-heated.
3. The wash buffer was prepared as follows:

To prepare 750 ml wash buffer – 30 ml of the concentrated wash buffer was diluted with 720 ml distilled water.

4. The standard working solution was prepared as follows:

The standard was centrifuged– 10000xg (60 seconds). 1 ml of reference standard and 1 ml of sample diluent (allowed to stand for 10 minutes) was added and overturned gently a few times. Once fully dissolved, the solution was carefully mixed with a pipette. This produced a 100 µg/ml working solution. Serial dilutions were prepared (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0 µg/ml was recommended). A visual demonstration of the dilution is presented in figure 5. 7 EP tubes were prepared and 500 µl of reference standard and sample diluent were added to each one. 500 µl of the 100 µg/ml working solution was pipetted to the first EP tube and mix thoroughly to produce a 50 µg/ml working solution. This process was proceeded by pipetting 500 µl from the former EP tube to the next. The last EP tube served as a blank.



**Figure 5:** Dilution method for working solution (figure was taken from the manual provided by the manufacturer).

5. The Biotinylated Detection Antibody working solution was prepared as follows:

The exact amount was calculated before the experiment (50 µl/well). The stock tube was centrifuged. 1x working solution with Biotinylated Detection Antibody Diluent was diluted with 100x Concentrated Biotinylated Detection Antibody.

6. The concentrated HRP Conjugate working solution was prepared as follows:

The exact amount was calculated before the experiment (100 µl/well). 1x working solution with Concentrated HRP Conjugate Diluent was diluted with 100x Concentrated HRP Conjugate.

### C.2.5 Assay procedure

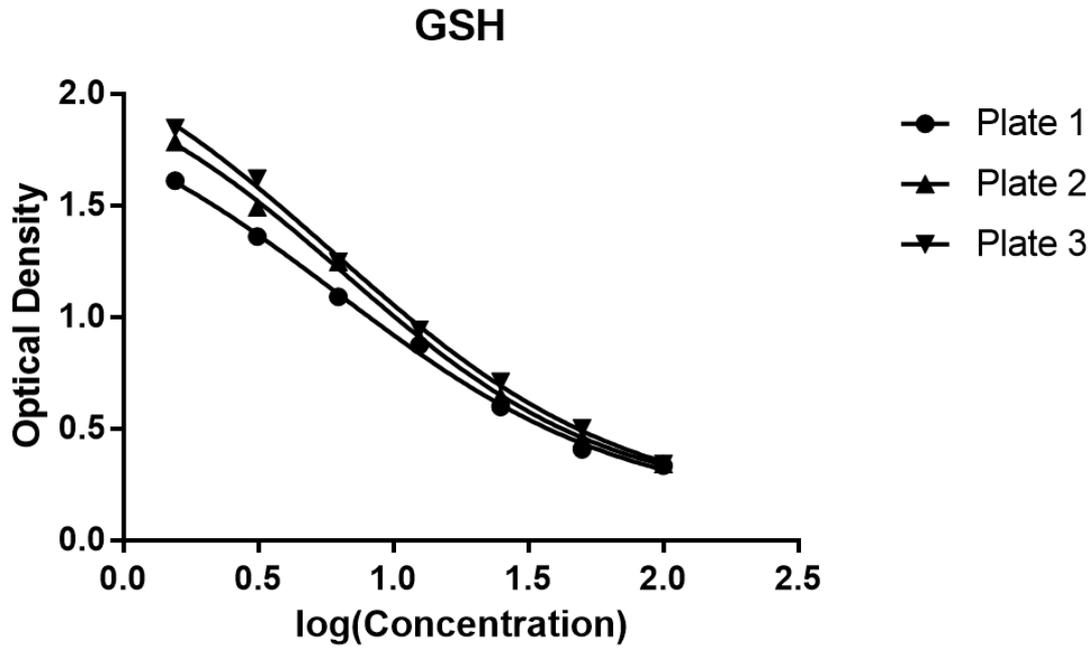
1. The standard working solution (50 µl) was added in the first two columns. All concentrations of the solution were added in duplicate.

2. A sample (50 µl) was added to each individual well, also in duplicate.

3. The biotinylated Detection Antibody (50  $\mu$ l) was added immediately to each individual well.
4. The plates were covered with one of the provided sealers and incubated (45 minutes - 37°C). SHAKER DTS-4 was used as an incubator.
5. The plates were aspirated and washed three times. This was done by adding the buffer (350  $\mu$ l) to each well and allowing them to soak for 2 minutes before decanting the buffer. After this step, the plates were patted dry against absorbent paper. The plates were washed automatically by a microplate washer (BioTek) in the laboratory.
6. Addition of HRP Conjugate (100  $\mu$ l) to each individual well.
7. The plates were covered with one of the provided sealers and incubated (30 minutes - 37°C).
8. The plates were aspirated and washed five times, as described in step 5.
9. The substrate reagent (90  $\mu$ l) was added to each individual well.
10. The plates were covered with one of the provided sealers and incubated (15 minutes - 37°C).
11. The stop solution (50  $\mu$ l) was added to each individual well.
12. The OD value (450 nm) was determined immediately. This was done by using a microplate reader.
13. The results were calculated.

#### **C.2.6 Calculation of results**

The average of each sample and standard were calculated since assays were performed in duplicate. Computer software capable of plotting a four-parameter logistic curve was used. The X-axis represented the standard concentration and the y-axis the OD values. See figure 6 for the logistic curve produced by the GSH concentration in this experiment for three plates. Three plates were needed as 96 samples had to be analysed in duplicate. Please see figure 7 for a detailed plate layout for all three plates used.



**Figure 6:** Standard logistic curve for glutathione (GSH) measured in plasma.

Rat sample allocation:

- 1 – 12 = SAL – OO – SAL group
- 13 – 24 = SAL – OO – NAC group
- 25 – 36 = SAL – EFV – SAL group
- 37 – 48 = SAL – EFV – NAC group
- 49 – 60 = LPS – OO – SAL group
- 61 – 72 = LPS – OO – NAC group
- 73 – 84 = LPS – EFV – SAL group
- 85 – 96 = LPS – EFV – NAC group

**Plate 1** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
<b>A</b>	0	0	1	1	9	9	17	17	25	25	33	33
<b>B</b>	1,56	1,56	2	2	10	10	18	18	26	26	34	34
<b>C</b>	3,13	3,13	3	3	11	11	19	19	27	27	35	35
<b>D</b>	6,25	6,25	4	4	12	12	20	20	28	28	36	36
<b>E</b>	12,5	12,5	5	5	13	13	21	21	29	29	37	37
<b>F</b>	25	25	6	6	14	14	22	22	30	30	38	38
<b>G</b>	50	50	7	7	15	15	23	23	31	31	39	39
<b>H</b>	100	100	8	8	16	16	24	24	32	32	40	40

**Plate 2** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
A	0	0	41	41	49	49	57	57	65	65	73	73
B	1,56	1,56	42	42	50	50	58	58	66	66	74	74
C	3,13	3,13	43	43	51	51	59	59	67	67	75	75
D	6,25	6,25	44	44	52	52	60	60	68	68	76	76
E	12,5	12,5	45	45	53	53	61	61	69	69	77	77
F	25	25	46	46	54	54	62	62	70	70	78	78
G	50	50	47	47	55	55	63	63	71	71	79	79
H	100	100	48	48	56	56	64	64	72	72	80	80

**Plate 3** – Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
A	0	0	81	81	89	89						
B	1,56	1,56	82	82	90	90						
C	3,13	3,13	83	83	91	91						
D	6,25	6,25	84	84	92	92						
E	12,5	12,5	85	85	93	93						
F	25	25	86	86	94	94						
G	50	50	87	87	95	95						
H	100	100	88	88	96	96						

**Figure 7:** Plate 1- 3 layout for rat glutathione (GSH) measured in plasma.

### Brain dissection method

The brain dissection followed after the rats were euthanised via decapitation on post-natal day 73. The brains were dissected into the FC, striatum and cerebellum. In short, the cerebellum was dissected first. Thereafter, the brain was dissected into the right- and left cerebral hemispheres, and the olfactory bulbs were removed. The FC was dissected first, according to a method set out by (Toua et al., 2010), thereafter the remaining parts of the brain were placed on the ventral side and the striatum was resected, using the external walls of the lateral ventricles as internal limits and the corpus callosum as external limit (Toua et al., 2010). These brain regions were snap frozen in liquid nitrogen and stored at -80°C (LAMB, North-West University) until the day of analysis. On the day of assay, the brain tissue was prepared according to the appropriate methods (explained individually for each assay).

### **C.3 c-Fos (Cerebellum)**

#### **C.3.1 Introduction**

c-Fos is an immediate early gene which serves as an excellent indirect measure of neuronal activity (Gallo et al., 2018). A previous pre-clinical study applied ELISA kits to investigate c-Fos concentrations within the brain (Pellegrino and Stork, 2006). In vitro quantitative determination of Rat c-Fos in this study was analysed by using the Rat c-Fos ELISA kit catalog No: E0046Ra from Bioassay Technology Laboratory (China).

#### Specification

- Sensitivity: 0.03 ng/ml
- Standard Curve Range: 0.05 - 30 ng/ml
- Precision – Intra-Assay: Coefficient of variation is <8%.

Precision – Inter-Assay: Coefficient of variation is <10%.

#### Principle of test

Bioassay Technology Laboratory's Rat c-Fos ELISA kit was a sandwich kit. The plates were pre-coated with Rat c-Fos antibody. c-Fos present in the sample was added and bonded to the antibodies coated on the wells. Biotinylated Rat c-Fos Antibody was then added and bonded to c-Fos in the sample. Streptavidin-Horseradish HRP was added and bonded to the Biotinylated c-Fos antibody. After incubation the unbound Streptavidin-HRP was washed away during the washing step. The substrate solution was added and colour developed in proportion to the amount of Rat c-Fos. This reaction was terminated by adding an acidic stop solution and absorbance was measured at 450 nm.

#### **C.3.2 Materials**

#### Provided materials:

- Manual
- Zipper bag (1 piece)
- Pre-coated ELISA plate (8 wells x 12 strips)
- Plate sealer (2 pieces)
- Standard Solution (1 vial 0.5 ml – 32 ng/ml)
- Standard Diluent (1 vial 3 ml)
- Streptavidin-HRP (1 vial 6 ml)
- Stop solution (1 vial 6 ml)
- Substrate Solution A (1 vial 6 ml)

- Substrate Solution B (1 vial 6 ml)
- Wash Buffer Concentrate (1 vial 20 ml – 30x)
- Biotinylated Rat c-Fos Antibody (1 vial 1 ml)

Other materials required:

- Microplate reader with 450nm wavelength filter BioTek FL600  
Microplate Fluorescence reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA)
- Incubator (37°C) SHAKER DTS-4
- High-precision transfer pipette
- EP tubes and disposable pipette tips
- Distilled water
- Absorbent paper

**C.3.3 Sample preparation**

1. The cerebellum tissue was rinsed with ice-cold phosphate buffered solution (PBS) (pH 7.4), weighed and homogenised in 0.5 ml PBS by sonication.
2. The homogenates were centrifuged (2000-3000 RPM, 20 minutes), thereafter the supernatant was collected and stored at -80°C until the next day of analysis.

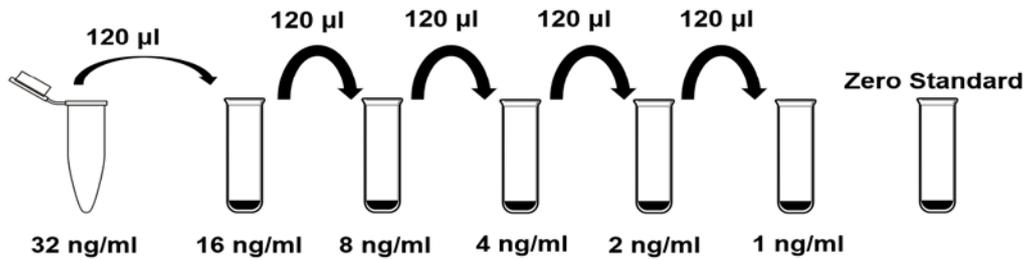
**C.3.4 Reagent preparation**

1. All reagents were brought to room temperature before use.
2. The standard was prepared  
120µl of the standard (32 ng/ml) was reconstituted with 120 µl of standard diluent to generate a 16 ng/ml standard stock solution. The standard was allowed to sit for 15 minutes before dilutions were made. Duplicate standard points were prepared by diluting the standard stock solution (16 ng/ml) 1:2 with the standard diluent to produce 8 ng/ml, 4ng/ml, 2 ng/ml and 1 ng/ml solutions. The standard diluent served as the zero standard (0 ng/ml). Dilution of standard solutions (Figure 8) suggested are as follows in table 1:

**Table 1:** Dilution of standard solutions

16 ng/ml	Standard No. 5	120 µl Original standard + 120 µl Standard diluent
18 ng/ml	Standard No. 4	120 µl Original standard No. 5 + 120 µl Standard diluent

4 ng/ml	Standard No. 3	120 µl Original standard No. 4 + 120 µl Standard diluent
2 ng/ml	Standard No. 2	120 µl Original standard No. 3 + 120 µl Standard diluent
1 ng/ml	Standard No. 1	120 µl Original standard No. 2 + 120 µl Standard diluent



**Figure 8:** Dilution method for standard solution.

3. The wash buffer was prepared

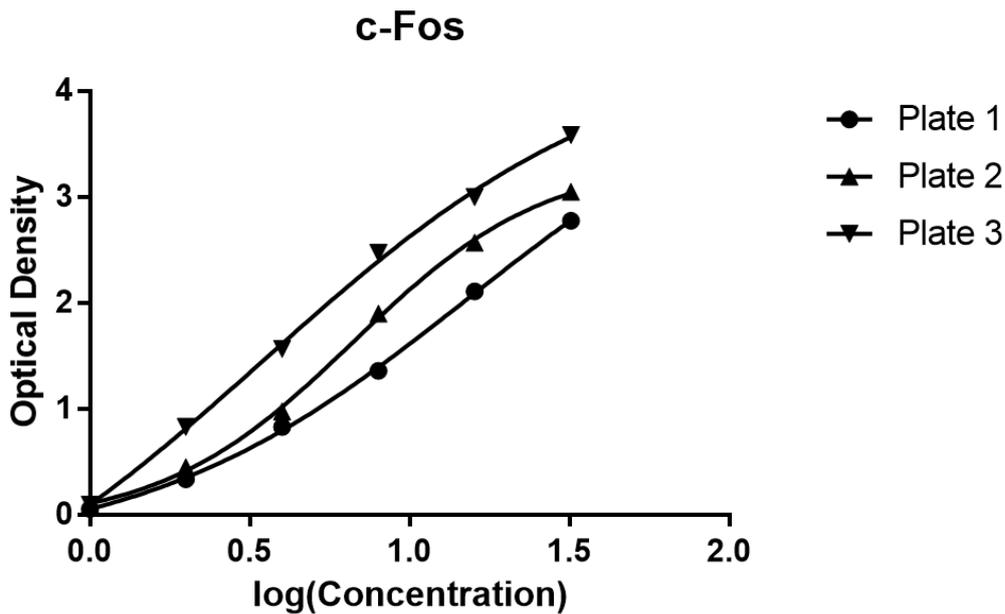
20 ml of wash buffer (concentrate 30x) was diluted with distilled water to yield 600 ml of 1x wash buffer.

### C.3.5 Assay procedure

1. All reagents, standard solutions and samples were prepared as previously described and brought to room temperature.
2. 50 µl standard was added to standard wells.
3. 40 µl sample and 10 µl anti-c-Fos antibody were added to the sample wells. 50 µl streptavidin-HRP was added to the sample- and standard wells (not blank control wells). The plates were covered with a sealer and incubated (60 minutes 37°C).
4. The plates were aspirated and washed five times. The plates were pat dry against paper towels.
5. 50 µl substrate solution A and 50 µl substrate solution B were added to each well. The plates were covered with a new sealer and incubated (10 minutes 37°C).
6. 50 µl stop solution was added to each well, the blue colour changed into yellow immediately.
7. The OD of each well was determined immediately by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

### C.3.6 Calculation of results

A standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis. These calculations were performed via a computer-based curve-fitting software and the best fit line was determined by regression analysis. See figure 9 for the logistic curve produced by the rat c-Fos concentration in this experiment for three plates. Three plates were needed as 96 samples had to be analysed in duplicate. Please see figure 10 for a detailed plate layout for all three plates used.



**Figure 9:** Standard logistic curve for rat c-Fos measured in the cerebellum.

Rat sample allocation;

- 1 – 12 = SAL – OO – SAL group
- 13 – 24 = SAL – OO – NAC group
- 25 – 36 = SAL – EFV – SAL group
- 37 – 48 = SAL – EFV – NAC group
- 49 – 60 = LPS – OO – SAL group
- 61 – 72 = LPS – OO – NAC group
- 73 – 84 = LPS – EFV – SAL group
- 85 – 96 = LPS – EFV – NAC group

**Plate 1** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
<b>A</b>	-	-	1	1	9	9	17	17	25	25	33	33
<b>B</b>	0	0	2	2	10	10	18	18	26	26	34	34
<b>C</b>	1	1	3	3	11	11	19	19	27	27	35	35
<b>D</b>	2	2	4	4	12	12	20	20	28	28	36	36
<b>E</b>	4	4	5	5	13	13	21	21	29	29	37	37
<b>F</b>	8	8	6	6	14	14	22	22	30	30	38	38
<b>G</b>	16	16	7	7	15	15	23	23	31	31	39	39
<b>H</b>	32	32	8	8	16	16	24	24	32	32	40	40

**Plate 2** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
<b>A</b>	-	-	41	41	49	49	57	57	65	65	73	73
<b>B</b>	0	0	42	42	50	50	58	58	66	66	74	74
<b>C</b>	1	1	43	43	51	51	59	59	67	67	75	75
<b>D</b>	2	2	44	44	52	52	60	60	68	68	76	76
<b>E</b>	4	4	45	45	53	53	61	61	69	69	77	77
<b>F</b>	8	8	46	46	54	54	62	62	70	70	78	78
<b>G</b>	16	16	47	47	55	55	63	63	71	71	79	79
<b>H</b>	32	32	48	48	56	56	64	64	72	72	80	80

**Plate 3** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
<b>A</b>	-	-	81	81	89	89						
<b>B</b>	0	0	82	82	90	90						
<b>C</b>	1	1	83	83	91	91						
<b>D</b>	2	2	84	84	92	92						
<b>E</b>	4	4	85	85	93	93						
<b>F</b>	8	8	86	86	94	94						
<b>G</b>	16	16	87	87	95	95						
<b>H</b>	32	32	88	88	96	96						

**Figure 10:** Plate 1- 3 layout for rat c-Fos measured in the cerebellum.

## **C.4 DAT (FC & striatum)**

### **C.4.1 Introduction**

DAT serve as one of the key regulators of dopamine (DA) in the central nervous system (Chen and Reith, 2000) as it is responsible for DA uptake (Lohr et al., 2017). In vitro quantitative determination of DAT in this study was analysed by using the rat DAT (Dopamine Transporter) ELISA kit catalog No: E-EL-R0343 from Elabscience (USA).

#### Certificate of analysis

- Production No.: E-EL-R0343
- Lot No.: K8A2CT9FD5
- Date: 2019-02-13

#### Specification

- Sensitivity: 0.19 ng/ml
- Detection Range: 0.31-20 ng/ml
- Specificity: DAT in given sample. No significant interference or cross-reactivity between DAT and analogues was found.
- Repeatability: Coefficient of variation is <10%.

#### Principle of test

Elabscience's ELISA kit used the Sandwich-ELISA principle. The micro ELISA plates provided were pre-coated with an antibody specific to Rat DAT. Standards or samples were added to the micro ELISA plate wells and combined with the specific antibody. A biotinylated detection antibody specific for Rat DAT and Avidin-HRP conjugate were added successively to each micro plate well and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contained Rat DAT, biotinylated detection Antibody and Avidin-HRP conjugate appeared blue in colour. The enzyme-substrate reaction was terminated by the addition of the stop solution and the colour turned to yellow. The OD was measured spectrophotometrically at a wavelength of 450 nm. The OD value was proportional to the concentration of Rat DAT. The concentration of Rat DAT in the samples was calculated by comparing the OD of the samples to the standard curve.

### **C.4.2 Materials**

#### Provided materials:

- Manual

- Certificate of Analysis
- Micro ELISA plate (8 wells x 12 strips)
- Plate sealer (5 pieces)
- Reference Standard (2 vials)
- Concentrated Biotinylated Detection Antibody (100x – 1 vial 120 µl)
- Concentrated HRP Conjugate (100x – 1 vial 120 µl)
- Reference standard & Sample diluent (1 vial 20 ml)
- Biotinylated Detection Antibody Diluent (1 vial 14 ml)
- HRP Conjugate Diluent (1 vial 14 ml)
- Concentrated Wash buffer (25x – 1 vial 30 ml)
- Substrate reagent (1 vial 10 ml)
- Stop solution (1 vial 10 ml)

Other materials required:

- Microplate reader with 450 nm wavelength filter BioTek FL600  
Microplate Fluorescence reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA)
- Incubator (37°C) SHAKER DTS-4
- High-precision transfer pipette
- EP tubes and disposable pipette tips
- Distilled water
- Absorbent paper
- Loading slot for wash buffer BioTek

### **C.4.3 Sample preparation**

1. The day before analysis, frontal cortical and striatal tissue was rinsed with ice-cold PBS (0.01M, pH 7.4), weighed and homogenised in PBS (tissue weight (g): PBS (ml) volume ratio = 1:9) by sonication. See table 2 (FC) and table 3 (striatum) for an indication on all brain tissue weights with appropriate PBS volume used.
2. The homogenates were centrifuged (5000xg, 5 minutes), thereafter the supernatant was collected and stored at -80°C until the next day of analysis.

**Table 2:** Weight of frontal cortical tissue with appropriate phosphate buffered solution (PBS) volume used.

Group	Rat ID	Tissue weight (g)	PBS volume (ml) (1g:9ml)	Group	Rat ID	Tissue weight (g)	PBS volume (ml) (1g:9ml)
SAL – OO – SAL	1	99.9	0.89	LPS – OO – SAL	49	46	0.41
	2	72.9	0.65		50	31.4	0.28
	3	16.7	0.15		51	83.8	0.75
	4	57.1	0.51		52	44.3	0.4
	5	110.5	0.99		53	79.2	0.66
	6	68.8	0.62		54	45.7	0.41
	7	94.2	0.85		55	71.6	0.64
	8	56.3	0.51		56	33.1	0.3
	9	86.8	0.78		57	42	0.38
	10	82.6	0.74		58	81.1	0.73
	11	121.9	1.09		59	41.1	0.37
	12	76.7	0.69		60	37.6	0.34
SAL – OO – NAC	13	66.8	0.6	LPS – OO – NAC	61	42.6	0.38
	14	70.8	0.64		62	86.2	0.78
	15	64.3	0.58		63	89.6	0.81
	16	60.9	0.55		64	65.2	0.59
	17	102.6	0.92		65	76.7	0.69
	18	118.4	1.07		66	89.5	0.81
	19	64.6	0.58		67	39.4	0.34
	20	58.1	0.52		68	42	0.38
	21	81.3	0.73		69	59.1	0.53
	22	96.1	0.86		70	51.7	0.47
	23	36	0.32		71	38.6	0.35
	24	87.9	0.79		72	65.7	0.59
SAL – EFV – SAL	25	95.3	0.86	LPS – EFV – SAL	73	90.6	0.82
	26	85	0.77		74	66.3	0.6
	27	52.1	0.47		75	83.6	0.75
	28	64.1	0.58		76	51.6	0.46
	29	89.8	0.81		77	47.9	0.43
	30	65.2	0.59		78	80.5	0.72

Addendum C: Peripheral- and neurochemical analyses

	31	40	0.36		79	39.2	0.35
	32	57.4	0.52		80	40.9	0.37
	33	60	0.54		81	86.9	0.78
	34	64.2	0.58		82	60.2	0.54
	35	108.2	0.97		83	39.2	0.35
	36	23.3	0.21		84	38	0.34
SAL – EFV – NAC	37	106.5	0.96	LPS – EFV – NAC	85	26.8	0.24
	38	32.9	0.3		86	34.4	0.31
	39	101.5	0.91		87	79.1	0.71
	40	47.1	0.42		88	53.8	0.48
	41	108.9	0.98		89	78.7	0.71
	42	12.8	0.12		90	44.3	0.4
	43	105.7	0.95		91	70.6	0.64
	44	15.9	0.14		92	70.5	0.63
	45	86.7	0.78		93	49.6	0.45
	46	34.9	0.31		94	33.3	0.3
	47	86.7	0.78		95	49.7	0.45
	48	13	0.12		96	77	0.69

**Table 3:** Weight of striatal tissue with appropriate phosphate buffered solution (PBS) volume used.

Group	Rat ID	Tissue weight (g)	PBS volume (ml) (1g:9ml)	Group	Rat ID	Tissue weight (g)	PBS volume (ml) (1g:9ml)
SAL – OO – SAL	1	49.8	0.45	LPS – OO – SAL	49	42.8	0.39
	2	84.2	0.76		50	49	0.44
	3	32.5	0.29		51	57.1	0.51
	4	48.7	0.44		52	54	0.49
	5	97.8	0.88		53	48.4	0.44
	6	70.6	0.64		54	53.6	0.48
	7	88.5	0.79		55	57	0.51
	8	48.4	0.44		56	45.5	0.41
	9	76.2	0.55		57	57.8	0.52
	10	82.1	0.74		58	62.8	0.57

Addendum C: Peripheral- and neurochemical analyses

	11	72.7	0.65		59	39.5	0.36
	12	58.5	0.53		60	39	0.35
SAL – OO – NAC	13	87.6	0.79	LPS – OO – NAC	61	52.7	0.47
	14	60.7	0.55		62	55.2	0.49
	15	57.6	0.52		63	63.9	0.58
	16	55.1	0.49		64	37.9	0.34
	17	99.7	0.9		65	57.2	0.51
	18	99.7	0.9		66	43.5	0.39
	19	71.2	0.64		67	66.4	0.6
	20	60.2	0.54		68	79.3	0.71
	21	70.5	0.63		69	51.2	0.46
	22	79.5	0.72		70	49.9	0.45
	23	76.2	0.69		71	89.4	0.8
	24	72.2	0.65		72	50.1	0.45
SAL – EFV – SAL	25	90.8	0.82	LPS – EFV – SAL	73	38.4	0.35
	26	79.4	0.71		74	36.8	0.33
	27	47	0.42		75	72.7	0.65
	28	67.5	0.61		76	74.3	0.69
	29	57.3	0.52		77	55.4	0.5
	30	52.3	0.47		78	50.4	0.4
	31	44.2	0.4		79	39.3	0.35
	32	32.4	0.29		80	73.6	0.66
	33	39.8	0.36		81	42.7	0.38
	34	52.2	0.47		82	47.7	0.43
	35	88.4	0.8		83	71.9	0.65
	36	28.6	0.26		84	45.4	0.41
SAL – EFV – NAC	37	79.7	0.72	LPS – EFV – NAC	85	52.8	0.48
	38	71	0.64		86	49.5	0.45
	39	91.3	0.82		87	71.6	0.64
	40	61.5	0.55		88	57.5	0.52
	41	83.1	0.75		89	46.3	0.42
	42	24	0.22		90	37.9	0.34
	43	72.9	0.66		91	45.6	0.41
	44	51.4	0.46		92	50	0.45
	45	53.7	0.48		93	48.5	0.44

*Addendum C: Peripheral- and neurochemical analyses*

	46	59.7	0.54		94	47.3	0.43
	47	65	0.59		95	24	0.22
	48	57.6	0.52		96	61.8	0.56

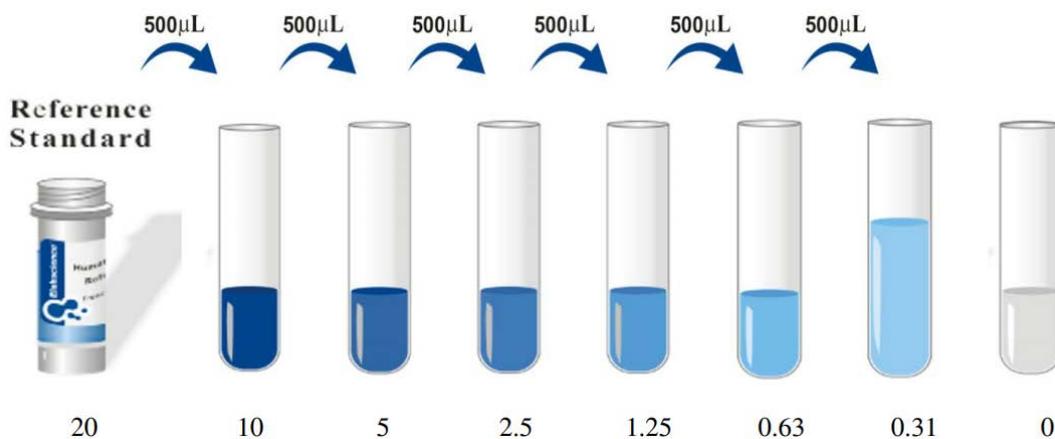
#### C.4.4 Reagent preparation

1. Before the procedure started, all reagents were brought to room temperature (18-25°C).
2. The microplate reader was pre-heated.
3. The wash buffer was prepared as follows:

To prepare 750 ml wash buffer – 30 ml of the concentrated wash buffer was diluted with 720 ml distilled water.

4. The standard working solution was prepared as follows:

The standard was centrifuged– 10000xg (60 seconds). 1 ml of reference standard and sample diluent (stand for 10 minutes) was added and overturned gently a few times. Once it was fully dissolved, it was carefully mixed with a pipette. This produced a 20 ng/ml working solution. Serial dilutions were prepared (20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0 ng/ml was recommended). A visual demonstration of the dilution is presented in figure 11. 7 EP tubes were prepared and 500 µl of reference standard and sample diluent were added to each one. 500 µl of the 20 ng/ml working solution was pipetted to the first EP tube and mix thoroughly to produce a 10 ng/ml working solution. This process was proceeded by pipetting 500 µl from the former EP tube to the latter EP tube. The last EP tube served as a blank.



**Figure 11:** Dilution method for working solution (figure was taken from the manual provided by the manufacturer).

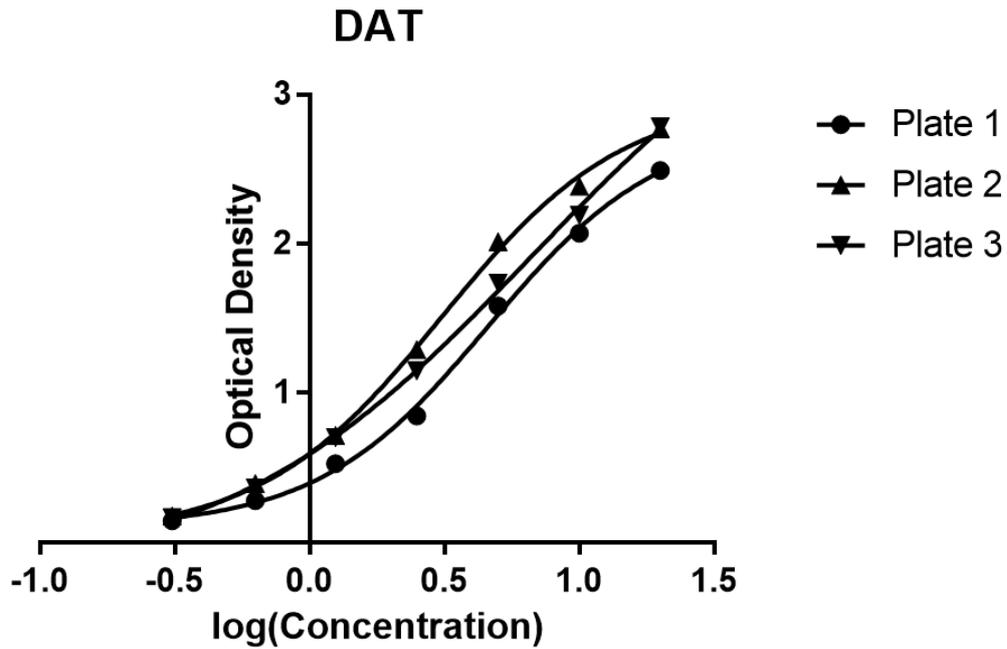
5. The Biotinylated Detection Antibody working solution was prepared  
The exact amount was calculated before the experiment (100 µl/well). The stock tube was centrifuged. 1x working solution with Biotinylated Detection Antibody Diluent was diluted with 100x Concentrated Biotinylated Detection Antibody.
6. The concentrated HRP Conjugate working solution was prepared  
The exact amount was calculated before the experiment (100 µl/well). 1x working solution with Concentrated HRP Conjugate Diluent was diluted with 100x Concentrated HRP Conjugate.

#### **C.4.5 Assay procedure**

1. The standard working solution (100 µl) was added in the first two columns. All concentrations of the solution were added in duplicate.
2. A sample (100 µl) was added to each individual well. With this assay all 96 samples from the FC were added to the first set of 96 wells, thereafter 96 samples of the striatum were added to the second set of 96 wells. Due to restricted number of wells these samples were not done in duplicate, but only a single sample was tested for each rat.
3. The plates were covered with a sealer and incubated (90 minutes 37°C). SHAKER DTS-4 were used as an incubator. The liquid from each well was removed.
4. The biotinylated Detection Antibody (50 µl) was added immediately to each individual well.
5. The plates were covered with one of the provided sealers and incubate (60 minutes - 37°C).
6. The plates were aspirated and washed three times. This was done by adding the buffer (350 µl) to each well and allowing them to soak for 2 minutes before decanting the buffer. After this step, the plates were patted dry against absorbent paper. The plates were washed automatically by a microplate washer (BioTek) in the laboratory.
7. Addition of HRP Conjugate (100 µl) to each individual well.
8. The plates were covered with one of the provided sealers and incubated (30 minutes - 37°C).
9. The plates were aspirated and washed five times, as described in step 6.
10. The substrate reagent (90 µl) was added to each individual well.
11. The plates were covered with one of the provided sealers and incubated (15 minutes - 37°C).
12. The stop solution (50 µl) was added to each individual well.
13. The OD value (450 nm) was determined immediately. This was done by using a microplate reader.
14. The results were calculated.

#### **C.4.6 Calculation of results**

Computer software capable of plotting a four-parameter logistic curve was used. The x-axis represented the standard concentration and the y-axis the OD values. See figure 12 for the logistic curve produced by the rat DAT concentration in this experiment for three plates. Three plates were needed as 96 samples had to be analysed. Please see figure 13 for a detailed plate layout for all three plated used.



**Figure 12:** Standard logistic curve for rat dopamine transporters (DAT) measured in the frontal cortex and striatum.

Rat sample allocation:

- 1 – 12 = SAL – OO – SAL group
- 13 – 24 = SAL – OO – NAC group
- 25 – 36 = SAL – EFV – SAL group
- 37 – 48 = SAL – EFV – NAC group
- 49 – 60 = LPS – OO – SAL group
- 61 – 72 = LPS – OO – NAC group
- 73 – 84 = LPS – EFV – SAL group
- 85 – 96 = LPS – EFV – NAC group

**Plate 1** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3 PFC	4	5	6	7	8	9	10	11	12
A	0	0	1	9	17	25	33	41	49	57	65	73
B	0,31	0,31	2	10	18	26	34	42	50	58	66	74
C	0,63	0,63	3	11	19	27	35	43	51	59	67	75
D	1,25	1,25	4	12	20	28	36	44	52	60	68	76
E	2,5	2,5	5	13	21	29	37	45	53	61	69	77
F	5	5	6	14	22	30	38	46	54	62	70	78
G	10	10	7	15	23	31	39	47	55	63	71	79
H	20	20	8	16	24	32	40	48	56	64	72	80

**Plate 2** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5 STRIATUM	6	7	8	9	10	11	12
A	0	0	81	89	1	9	17	25	33	41	49	57
B	0,31	0,31	82	90	2	10	18	26	34	42	50	58
C	0,63	0,63	83	91	3	11	19	27	35	43	51	59
D	1,25	1,25	84	92	4	12	20	28	36	44	52	60
E	2,5	2,5	85	93	5	13	21	29	37	45	53	61
F	5	5	86	94	6	14	22	30	38	46	54	62
G	10	10	87	95	7	15	23	31	39	47	55	63
H	20	20	88	96	8	16	24	32	40	48	56	64

**Plate 3** – Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3	4	5	6	7	8	9	10	11	12
A	0	0	65	73	81	89						
B	0,31	0,31	66	74	82	90						
C	0,63	0,63	67	75	83	91						
D	1,25	1,25	68	76	84	92						
E	2,5	2,5	69	77	85	93						
F	5	5	70	78	86	94						
G	10	10	71	79	87	95						
H	20	20	72	80	88	96						

**Figure 13:** Plate 1- 3 layout for rat dopamine transporters (DAT) measured in the frontal cortex and striatum.

## **C.5 PPP1R1B (FC & striatum)**

### **C.5.1 Introduction**

PPP1R1B (Wang et al., 2017) serves as a valuable marker for DA projections (Petryszyn et al., 2017). In vitro quantitative determination of PPP1R1B in this study was analysed by using the Rat Protein Phosphatase 1 Regulatory subunit 1B (PPP1R1B) ELISA kit catalog No: MBS7245452 from MyBioSource.

#### Specification

- Sensitivity: 0.1 ng/ml
- Specificity: PPP1R1B in given sample. No significant interference or cross-reactivity between PPP1R1B and analogues was found.

- Repeatability:

In the same lot - Coefficient of variation is 4.2 – 5.6%

In different lots - Coefficient of variation is 6.5 – 8.1%

- Spike recovery: 94 – 103%

- Linearity range:

1:1 – 94 – 106%

1:2 – 93 - 102%

1:4 – 92 – 103%

1:8 – 96 – 102%

#### Principle of test

PPP1R1B ELISA kit applied the competitive enzyme immunoassay technique by utilizing a polyclonal anti-PPP1R1B antibody and an PPP1R1B-HRP conjugate. The assay sample and buffer were incubated together with the PPP1R1B-HRP conjugate in the pre-coated plates for one hour. After the incubation period, the wells were decanted and washed five times. The wells were then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction formed a blue coloured complex. Finally, the stop solution was added to stop the reaction, and the solution turned yellow. The intensity of colour was measured spectrophotometrically at 450nm in a microplate reader. The intensity of the colour was inversely proportional to the PPP1R1B concentration since PPP1R1B from samples and PPP1R1B-HRP conjugate competed for the anti-PPP1R1B antibody binding site. Since the number of sites was limited, and more sites were occupied by PPP1R1B from the sample, fewer sites were left to bind onto the PPP1R1B-HRP conjugate. A standard curve was plotted relating the intensity of the colour (OD) to the concentration of standards. The PPP1R1B concentration in each sample was interpolated into the standard curve.

## **C.5.2 Materials**

### Provided materials:

- Manual
- Micro ELISA plate (8 wells x 12 strips)
- Standard A (0 ng/ml 1 vial)
- Standard B (0.5 ng/ml 1 vial)
- Standard C (1.0 ng/ml 1 vial)
- Standard D (2.5 ng/ml 1 vial)
- Standard E (5.0 ng/ml 1 vial)
- Standard F (10 ng/ml 1 vial)
- Enzyme Conjugate (1 vial 6 ml)
- Wash solution (100x – 1 vial 10 ml)
- Substrate A (1 vial 6 ml)
- Substrate B (1 vial 6 ml)
- Stop solution (1 vial 6 ml)
- Balance solution (1 vial 3 ml)

### Other materials required:

- Microplate reader with 450 nm wavelength filter BioTek FL600  
Microplate Fluorescence reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA)
- Incubator (37°C) SHAKER DTS-4
- High-precision transfer pipette
- EP tubes and disposable pipette tips
- Distilled water
- Absorbent paper
- Loading slot for wash buffer BioTek

## **C.5.3 Sample preparation**

1. The day before analysis, the frontal cortical and striatal tissue was rinsed with ice-cold PBS buffer (0.02M, pH 7.0-7.2) and weighed.
2. To comply with the method set out by MyBioSource as well as the matter of getting enough supernatant, two samples from the same behavioural cohort were pooled together. The tissues were then homogenised in PBS (tissue weight (g): PBS (ml) volume ratio = 1:1) by sonication. See table 4 (FC) and 5 (striatum) for tissue weight and appropriate PBS volume used.

3. The homogenates were centrifuged (1500xg, 15 minutes), thereafter the supernatant was collected and stored at -80°C until the next day of analysis.

**Table 4:** Weight of frontal cortical tissue with appropriate phosphate buffered solution (PBS) volume used.

Group	Pooled sample	Rat ID	Tissue weight (mg)	PBS volume (µl) (1mg:1µl)	Group	Pooled sample	Rat ID	Tissue weight (mg)	PBS volume (µl) (1g:1ml)
SAL – OO – SAL	1	1	89.3	114.7	LPS – OO – SAL	25	49	34	98
		3	25.4				58	64.1	
	2	2	73.2	120.5		26	54	50.9	88.3
		4	47.3				57	37.4	
	3	5	91	173		27	51	79.1	101.8
		6	82				60	22.7	
	4	7	94	136.2		28	53	78.8	109.5
		8	42.2				59	30.7	
	5	9	66.7	169.9		29	50	34.3	106.7
		10	102.9				55	72.4	
	6	11	107.8	180.6		30	52	37.5	77.9
		12	72.8				56	40.3	
SAL – OO – NAC	7	13	72.8	162.2	LPS – OO – NAC	31	61	37.6	124.4
		24	89.4				62	86.8	
	8	14	54.4	120.5		32	63	71.8	114.2
		15	66.1				71	30.1	
	9	16	22.7	122.5		33	64	68.5	130.7
		17	99.8				65	62.2	
	10	18	72.3	119.7		34	66	73	115.3
		19	47.4				67	42.3	
	11	20	49.5	120.5		35	68	55.5	104.6
		21	71				69	49.1	
	12	22	77	119.9		36	70	42.4	103.4
		23	42.9				72	73.1	
SAL – EFV – SAL	13	25	94.4	114.9	LPS – EFV – SAL	37	73	88	120.7
		27	20.5				74	32.7	
	14	26	50.8	102.6		38	75	77.9	111.6
		31	51.8				80	33.7	
	15	28	73.8	174.4		39	76	68.9	111.2
		29	100.6				77	42.3	

Addendum C: Peripheral- and neurochemical analyses

	16	30	76.5	103.3		40	78	78.3	106.5		
		32	26.8				79	28.2			
	17	33	75.3	119.3		41	81	84.5	134.4		
		34	44				82	49.9			
	18	35	95.2	126.7		42	83	49.6	129.1		
		36	31.5				84	79.5			
	SAL – EFV – NAC	19	37	98.3		105.7	LPS – EFV – NAC	43	85	66.2	117.7
			38	7.4					86	51.5	
20		39	91.2	144.9	44	87		82.6	129.6		
		40	53.7			88		47			
21		41	112.1	142.7	45	89		60.6	101.2		
		42	30.6			90		40.6			
22		43	68.6	109.6	46	91		76.1	119.3		
		44	41			93		43.2			
23		45	75.5	131.1	47	92		76.1	102.2		
		46	55.6			95		26.1			
24		47	82.5	126.8	48	94		72.8	140.7		
		48	44.3			96		67.9			

**Table 5:** Weight of striatal tissue with appropriate phosphate buffered solution (PBS) volume used.

Group	Pooled sample	Rat ID	Tissue weight (mg)	PBS volume (µl) (1mg:1µl)	Group	Pooled sample	Rat ID	Tissue weight (mg)	PBS volume (µl) (1mg:1µl)
SAL – OO – SAL	1	2	101.1	142	LPS – OO – SAL	25	52	26.1	113.4
		3	40.9				57	73.8	
	2	1	62.6	104.3		26	55	66.5	106.1
		4	41.7				59	39.6	
	3	5	91.5	170.4		27	49	58.6	119.7
		6	78.9				50	61.1	
	4	7	77.1	120.9		28	51	57.9	111.8
		8	43.8				53	53.9	
	5	9	72.9	162.3		29	54	61.8	116.7
		10	89.4				56	54.9	

Addendum C: Peripheral- and neurochemical analyses

	6	11	75.9	145.1		30	58	56.5	109.2
		12	69.2				60	52.7	
SAL – OO – NAC	7	22	30.4	111.1	LPS – OO – NAC	31	62	71.6	107.6
		24	80.7				67	36	
	8	15	72.1	102.7		32	69	67.6	106.6
		20	30.6				70	39	
	9	17	70.1	106.5		33	64	44.1	105
		23	36.4				68	60.9	
	10	16	69.7	108.6		34	65	47.7	104.8
		19	38.9				66	57.1	
	11	14	46.7	113.6		35	63	49.7	109.6
		21	66.9				72	59.9	
12	13	50.1	101	36	61	52.3	102.5		
	18	50.9			71	50.2			
SAL – EFV – SAL	13	30	92.5	126.5	LPS – EFV – SAL	37	82	43.1	132.9
		34	34				83	89.8	
	14	29	87.4	123.2		38	74	37.3	110.9
		32	35.8				84	73.6	
	15	25	86.6	131.6		39	79	68	113.4
		31	44.2				81	45.4	
	16	27	46.6	115.1		40	73	60.5	123.4
		35	68.5				80	62.9	
	17	28	64.3	111.3		41	75	52.5	105.1
		36	47				76	52.6	
18	26	56.7	120.3	42	77	46.3	112.8		
	33	63.6			78	66.5			
SAL – EFV – NAC	19	37	85.6	132	LPS – EFV – NAC	43	85	79.5	107.9
		38	46.4				88	28.4	
	20	39	99.8	131		44	86	74.2	118.6
		48	31.2				90	44.4	
	21	40	50.6	141.3		45	92	45.4	124.4
		41	90.7				96	79	
	22	42	68.8	145.7		46	93	54.7	100
		43	76.9				94	45.3	
	23	44	70.4	140.5		47	89	48.2	95.2

*Addendum C: Peripheral- and neurochemical analyses*

		45	70.1			95	47	
	24	46	53.2	109.2		87	45.7	94.9
		47	56			48	91	

#### **C.5.4 Reagent preparation**

1. All kit components and samples were brought to room temperature (20-25°C).
2. The samples were prepared as follows:

The concentration was predicted before starting the assay. The concentrations were unknown and there for a preliminary experiment was done to determine the optimal dilution.

3. The wash solution was prepared as follows:

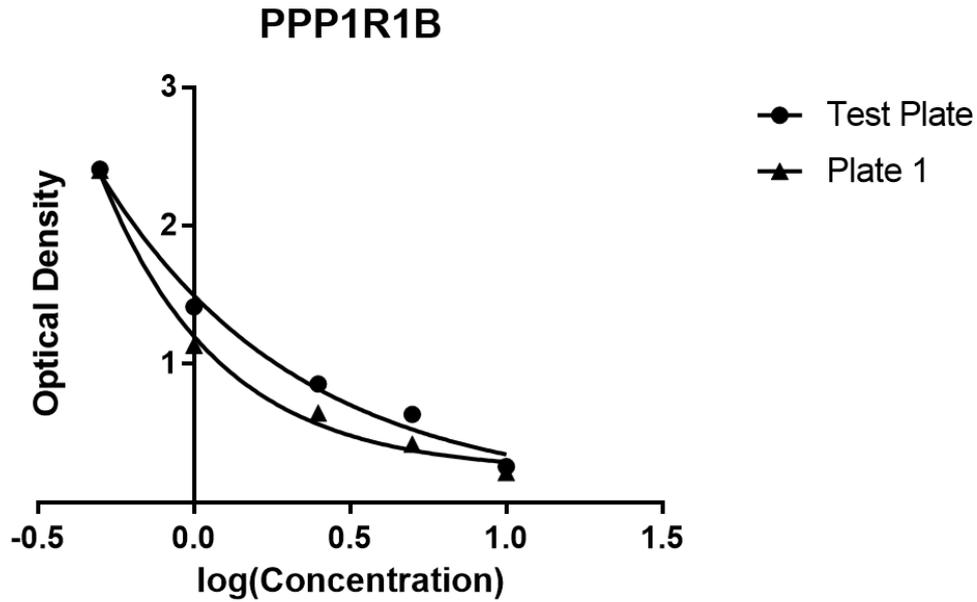
10 mL of wash solution concentrate (100x) was diluted with 990 mL of distilled water to prepare 1000 mL of wash solution (1x).

#### **C.5.5 Assay procedure**

1. A preliminary experiment was performed.
2. 100 µL of standards and samples were added to the appropriate wells. 100 µL of PBS (pH 7.0-7.2) were added in the blank control well. With this assay (as previously explained) 2 brain samples were pooled together to generate one sample. With this assay all 48 samples from the FC were added to the first set of 48 wells, thereafter 48 samples of the striatum were added to the second set of 48 wells. Due to restricted samples sizes these samples were not done in duplicate.
3. 10 µL of the balance solution was dispensed into 100 µL samples and mixed well.
4. 50 µL of conjugate was added to each well (NOT blank control well). The plates were covered and incubated (60 minutes at 37°C). SHAKER DTS-4 were used as an incubator.
5. The plates were aspirated and washed five times. The washer was set for a soaking time of 10 seconds and shaking time of 5 seconds between each wash.
6. After washing, the plates were pat dry against absorbent paper.
7. 50 µL Substrate A and 50 µL Substrate B were added to each well, including the blank control well. The plates were covered and incubated (15-20minutes at 37°C).
8. 50 µL of Stop Solution was added to each well including blank control well.
9. The OD was determined at 450 nm using a microplate reader.

#### **C.5.6 Calculation of results**

A standard curve was constructed by plotting the average OD for each standard on the horizontal (X) axis against the concentration on the vertical (Y) axis, and the best fit curve were drawn using statistical software to generate a four-parameter logistic curve-fit. An x-axis for the OD and a y-axis for the concentration is also a choice. The data may be linearized by plotting the log of the concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. Calculate the concentration of samples corresponding to the mean absorbance from the standard curve (Figure 14).



**Figure 14:** Standard logistic curve for rat phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B) measured in the frontal cortex and striatum.

Rat sample allocation:

- 1 – 6 = SAL – OO – SAL group
- 7 – 12 = SAL – OO – NAC group
- 13 – 18 = SAL – EFV – SAL group
- 19 – 24 = SAL – EFV – NAC group
- 25 – 30 = LPS – OO – SAL group
- 31 – 36 = LPS – OO – NAC group
- 37 – 42 = LPS – EFV – SAL group
- 43 – 48 = LPS – EFV – NAC group

**Test Plate** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3 PFC	4	5 STRIATUM	6	7	8	9	10	11	12
A	-	-	1	1								
B	0	0	7	7								
C	0,5	0,5	13	13								
D	1	1	19	19								
E	2,5	2,5	25	25								
F	5	5	31	31								
G	10	10	37	37								
H	10	10	43	43								

**Plate 1** - Sample numbers indicated in appropriate block/well of plate used.

	1 Std.	2 Std.	3 PFC	4	5	6	7	8 STRIATUM	9	10	11	12
A	-	-	2	11	21	30	40	2	11	21	30	40
B	0	0	3	12	22	32	41	3	12	22	32	41
C	0,5	0,5	4	14	23	33	42	4	14	23	33	42
D	1	1	5	15	24	34	44	5	15	24	34	44
E	2,5	2,5	6	16	26	35	45	6	16	26	35	45
F	5	5	8	17	27	36	46	8	17	27	36	46
G	10	10	9	18	28	38	47	9	18	28	38	47
H	10	10	10	20	29	39	48	10	20	29	39	48

**Figure 15:** Test plate and plate 1 layout for rat phosphoprotein phosphatase-1 regulatory subunit 1B (PPP1R1B) measured in the frontal cortex and striatum.

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**ADDENDUM D**

**AUTHOR GUIDELINES: FRONTIERS IN PSYCHIATRY**

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Supplementary material is not typeset so please ensure that all information is clearly presented, the appropriate caption is included in the file and not in the manuscript, and that the style conforms to the rest of the article. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, affiliations or correspondence in the supplementary files. For Supplementary Material templates (LaTeX and Word) see ([http://www.frontiersin.org/design/zip/Frontiers\\_Supplementary\\_Material.zip](http://www.frontiersin.org/design/zip/Frontiers_Supplementary_Material.zip)).

#### *Suggested Fonts*

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.

Headings and subheadings need to be defined in Times New Roman, 12, bold.

The text of the abstract section should be in 12 point normal Times New Roman.

The body text is in 12 point normal Times New Roman.

#### D.2.3.5. File Requirements

Figures should be included in the provided pdf. In case of acceptance, our Production Office might require (<https://www.frontiersin.org/about/author-guidelines> \l "ResolutionRequirements" \t) of the figures included in the manuscript in eps, jpg or tif format. In order to be able to upload more

than one figure at a time, save the figures (labelled in order of appearance in the manuscript) in a zip file, and upload them as 'Supplementary Material Presentation'.

To facilitate the review process, please include a Word Count at the beginning of your manuscript, one option is teXcount which also has an online interface.

During the Interactive Review, authors are encouraged to upload versions using 'Track Changes'. Editors and Reviewers can only download the PDF file of the submitted manuscript.

#### D.2.3.6. Additional Requirements per article types

##### D.2.3.6.1. CrossMark Policy

(<http://www.crossref.org/crossmark/index.html>) is a multi-publisher initiative to provide a standard way for readers to locate the current version of a piece of content. By applying the CrossMark logo Frontiers is committing to maintaining the content it publishes and to alerting readers to changes if and when they occur. Clicking on the CrossMark logo will tell you the current status of a document and may also give you additional publication record information about the document.

##### D.2.3.6.2. Commentaries on Articles

For General Commentaries, the title of your manuscript must have the following format: "Commentary: Title of the original article". At the beginning of your Commentary, please provide the complete citation of the article commented on. Authors commenting on a Frontiers article must submit their commentary for consideration to the same Journal and Specialty as the original article.

Rebuttals may be submitted in response to Commentaries; our limit in place is one commentary and one response. Rebuttals should be submitted as General Commentary articles and the title should have the following format: "Response: Commentary: Title of original article".

#### D.2.4. Figure and Table Guidelines

##### D.2.4.1. CC-BY Licence

All figures, tables, and images will be published under the following - (<https://creativecommons.org/licenses/by/4.0/>) and permission must be obtained for use of copyrighted material from other sources (including re-published/adapted/modified/partial figures and images from the internet). It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

#### D.2.4.2. General Style Guidelines for Figures

The maximum number of figures and tables for all article types are shown in the summary table. Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript, the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order.

For graphs, there must be a self-explanatory label (including units) along each axis. For figures with more than one panel, panels should be clearly indicated using labels (A), (B), (C), (D), etc. However, do not embed the part labels over any part of the image, these labels will be added during typesetting according to Frontiers journal style. Please note that figures which are not according to the guidelines will cause substantial delay during the production process.

It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

#### D.2.4.3. General Style Guidelines for Tables

Tables should be inserted at the end of the manuscript. If you use a word processor, build your table in word. An empty line should be left before and after the table.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material on the online article abstract page at the time of acceptance. The author will be notified during the typesetting of the final article if this is the case. A link in the final PDF will direct to the online material.

#### D.2.4.4. Figure and Table Requirements

##### Legends

Legends should be preceded by the appropriate label, for example "Figure 1" or "Table 4". Figure legends should be placed at the end of the manuscript (for supplementary images you must include the caption with the figure, uploaded as a separate file). Table legends must be placed immediately before the table. Please use only a single paragraph for the legend. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

##### Image Size

Figure images should be prepared with the PDF layout in mind, individual figures should not be longer than one page and with a width that corresponds to 1 column or 2 columns.

*All articles are prepared using the 2 column layout: 2 column articles can contain images 85 mm or 180 mm wide.*

#### D.2.4.5. Format

The following formats are accepted:

TIFF (.tif) TIFF files should be saved using LZW compression or any other non-lossy compression method.

JPEG (.jpg)

EPS (.eps) EPS files can be uploaded upon acceptance

Color Image Mode

Images must be submitted in the color mode RGB.

Resolution Requirements

All images must be uploaded separately in the submission procedure and have a resolution of 300 dpi at final size. Check the resolution of your figure by enlarging it to 150%. If the resolution is too low, the image will appear blurry, jagged or have a stair-stepped effect.

Please note saving a figure directly as an image file (JPEG, TIF) can greatly affect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software. EPS files can be uploaded upon acceptance.

Legibility

Figures must be legible. Check the following:

- The smallest visible text is no less than 8 points in height, when viewed at actual size.
- Solid lines are not broken up.
- Image areas are not pixilated or stair stepped.
- Text is legible and of high quality.
- Any lines in the graphic are no smaller than 2 points width.

#### D.2.5. Funding disclosure

Details of all funding sources must be provided in the funding section of the manuscript including grant numbers, if applicable. All Frontiers articles are published with open access under the CC-BY Creative Commons attribution license. Articles published with Frontiers automatically fulfil or exceed the requirements for open access mandated by many institutions and funding bodies, including the National Institutes of Health, the Medical Research Council, Research Councils UK, and the Wellcome Trust. Frontiers submits funding data to the Open Funder Registry which is a funder identification service from CrossRef resulting from collaboration between scholarly publishers and funding agencies.

#### D.2.6. Materials and Data Policies

Frontiers is committed to open science and open data, we require that authors make available all data relevant to the conclusions of the manuscript. Generated data should be publicly available and cited in accordance with our (<https://www.frontiersin.org/about/author-guidelines>" \l "DataCitationGuidelines). We aim to achieve the best community standards regarding data availability, ensuring increased levels of transparency and reproducibility in our published articles.

Our policies on data availability are informed by community-driven standards, which Frontiers endorses, such as the (<https://cos.io/our-services/top-guidelines>) (TOP) guidelines, and the joint declaration of data citation principles produced by (<https://www.force11.org/group/joint-declaration-data-citation-principles-final>" \t).

##### D.2.6.1. Availability of Materials

Authors are required to make all materials used to conduct their research available to other researchers. Research materials necessary to enable the reproduction of an experiment should be clearly indicated in the Materials and Methods section. Relevant materials such as protocols, analytic methods, and study material should preferably be uploaded to an online repository providing a global persistent link/identifier. If this is not possible, authors are strongly encouraged to make this material available upon request to interested researchers, and this should be stated in the manuscript.

##### *Resource Identification Initiative*

Authors wishing to participate in the (<https://www.force11.org/group/resource-identification-initiative>) should cite antibodies, genetically modified organisms, software tools, data, databases, and services using the corresponding catalog number and RRID in your current manuscript. For more information about the project and for steps on how to search for an RRID, please click ([http://www.frontiersin.org/files/pdf/letter\\_to\\_author.pdf](http://www.frontiersin.org/files/pdf/letter_to_author.pdf)).

#### D.2.6.2. Availability of Data

Frontiers requires that authors make the “minimal data set” underlying the findings described and used to reach the conclusions of the manuscript, available to any qualified researchers. The data should be FAIR – findable, accessible, interoperable, and reusable – so that other researchers can locate and use the data. However, exceptions are granted if data cannot be made publicly available for legal or ethical reasons.

To comply with best practice in their field of research, authors are required to make certain types of data available to readers at time of publication in specific stable, community-supported repositories such as those listed below. Authors are encouraged to contact our data availability office at (<mailto:datapolicy@frontiersin.org>) prior to submission with any queries concerning data reporting.

##### D.2.6.2.1 Exceptions related to Availability of Data

We strongly encourage sharing the maximal amount of data, however where ethical, legal or privacy issues are present the data should not be shared. In cases where some or all data cannot be shared for legal, ethical or privacy restrictions, the authors should make these limitations clear in the Data Availability Statement at the time of submission.

#### D.2.6.3. Data Citation Guidelines

Authors are encouraged to cite all datasets generated or analyzed in the study. Where datasets are cited, they should be included in the (<https://www.frontiersin.org/about/author-guidelines>" \ "References") to maximize future usability. The following format should be used:

[Dataset] Author names. (year) Data Title. Repository name. Version. Persistent identifier

#### D.2.6.4. Data Availability Statements

During the submission process, authors will be asked to detail the location of the raw data underlying the conclusions made in the manuscript, and whether it will be made available to other researchers following publication. Authors will also be asked for the details of any existing datasets that have been analysed in the manuscript. These datasets should be cited in accordance with our data citation guidelines.

A statement will be automatically generated using the information provided in the submission form; however, manuscripts containing incomplete or incorrect statements will be prevented from entering the review process.

## Examples of acceptable statements

1. Datasets are in a publicly accessible repository:

The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF REPOSITORY] [LINK]

2. Datasets are available on request:

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

3. All relevant data is contained within the manuscript:

All datasets [GENERATED/ANALYZED] for this study are included in the manuscript/supplementary files.

### D.2.6.5. Mandatory data and required repositories

### D.2.7. Statistics

Frontiers requires that all statements concerning quantitative differences should be based on quantitative data and statistical testing. For example, if a quantitative statement is made regarding the abundance of a certain protein based on a western blot, we request that the blot be scanned and the abundance assessed quantitatively using the correct analytic software (e.g. ImageJ) and statistics in order to support that statement.

Statistics should/must be applied for independent experiments. The number of independent samples and the deviation parameters (e.g. Standard Error of the Mean, Standard Deviation, Confidence Intervals) should be clearly stated in the Methods or the Figure legends. In general, technical replicates within a single experiment are not considered to be independent samples. Where multiple comparisons are employed (e.g. microarray data or Genome-wide association studies), any analysis should correct for false positive results. Descriptions of statistical procedures should include the software and analysis used, and must be sufficiently detailed to be reproduced.

## **D.3. Editorial Policies and Publication Ethics**

Frontiers' ethical policies are a fundamental element of our commitment to the scholarly community. These policies apply to all the Frontiers in journal series. Frontiers has been a member of the Committee of Publication Ethics since January 2015 and follows COPE guidelines where applicable.

### D.3.1. Authorship and Author Responsibilities

Frontiers follows the guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed: (<http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.htm>)

- Substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work;
- Drafting the work or revising it critically for important intellectual content;
- Provide approval for publication of the content;
- Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Contributors, who do not meet these criteria, but nonetheless provided important contributions to the final manuscript should be included in the acknowledgements section. It is the authors responsibility to get written approval by persons named in the acknowledgement section. In order to provide appropriate credit to all authors, as well as assigning responsibility and accountability for published work, individual contributions should be specified as an Author Contributions statement. This should be included at the end of the manuscript, before the References. The statement should specify the contributions of all authors. You may consult the Frontiers manuscript guidelines for formatting instructions. Please see an example here:

AB, CDE and FG contributed conception and design of the study; AB organized the database; CDE performed the statistical analysis; FG wrote the first draft of the manuscript; HIJ, KL, AB, CDE and FG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

The corresponding author takes primary responsibility for communication with the journal and editorial office during the submission process, throughout peer review and during publication. The corresponding author is also responsible for ensuring that the submission adheres to all journal requirements including, but not exclusive to, details of authorship, study ethics and ethics approval, clinical trial registration documents and conflict of interest declaration. The corresponding author should also be available post-publication to respond to any queries or critiques.

Requests to modify the authors list after submission should be made to the editorial office using the ([http://www.frontiersin.org/files/pdf/Authorship\\_change\\_form.pdf](http://www.frontiersin.org/files/pdf/Authorship_change_form.pdf)).

### D.3.2. Research Integrity

Material submitted to Frontiers must comply with the following policies to ensure ethical publication of academic work:

- i. *Original content and duplicate publication*: Frontiers only publish original content. Authors confirm the submission of original content in the Terms & Conditions upon submission. Manuscripts submitted to Frontiers must not have been previously published or be under consideration for publication elsewhere, either in whole or in part. If an article has been previously submitted for publication elsewhere, Frontiers will only consider publication if the article has been definitively rejected by the other publisher(s) at the point of submission to Frontiers.
- ii. *Redundant publication*: Frontiers considers the submission and publication of very similar articles based on the same experiment or study to be unethical.
- iii. *Fabrication and falsification*: Frontiers oppose both the fabrication of data or images (i.e. fake or made up data) and the falsification of data or images (i.e. the intentional misrepresentation or deceptive manipulation of data).
- iv. *Plagiarism*: Plagiarism occurs when an author attempts to present previously published work as original content. Every manuscript submitted to Frontiers is screened for textual overlap by the software CrossCheck, powered by iThenticate. Manuscripts found to contain textual overlap are not considered for publication by Frontiers. For more details on what constitutes plagiarism, please see (<https://www.frontiersin.org/about/author-guidelines> \ "Plagiarism").

We reserve the right to contact the affiliated institutions of authors, who have not acted according to good research and publication practices.

### D.3.3. Plagiarism and Duplication

Frontiers checks all submitted manuscripts for plagiarism and duplication, and publishes only original content. Those manuscripts where plagiarism or duplication is shown to have occurred will not be considered for publication in a Frontiers journal. It is required that all submissions must consist as far as possible of content that has not been published previously. In accordance with ([http://publicationethics.org/files/International%20standards\\_authors\\_for%20website\\_11\\_Nov\\_2011.pdf](http://publicationethics.org/files/International%20standards_authors_for%20website_11_Nov_2011.pdf)), we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work.

For submissions adapted from theses, dissertations, conference abstracts or proceedings papers, please see the following sections for more information.

## Theses and Dissertations

Frontiers allows the inclusion of content which first appeared in an author's thesis so long as this is the only form in which it has appeared, is in line with the author's university policy, and can be accessed online. If the thesis is not archived online, it is considered as original unpublished data and thus is subject to the unpublished data restrictions of some of our article types. This inclusion should be noted in the Acknowledgements section of the manuscript and the thesis should be cited and referenced accordingly in the Reference list.

### D.3.4. Conflicts of Interest

A conflict of interest can be anything potentially interfering with, or that could reasonably be perceived as interfering with, full and objective peer review, decision-making or publication of articles submitted to Frontiers. Personal, financial and professional affiliations or relationships can be perceived as conflicts of interest.

All authors and members of Frontiers Editorial Boards are required to disclose any actual and potential conflicts of interest at submission or upon accepting an editorial or review assignment.

The Frontiers review system is designed to guarantee the most transparent and objective editorial and review process, and because handling editor and reviewers' names are made public upon the publication of articles, conflicts of interest will be widely apparent.

Failure to declare competing interests can result in the rejection of a manuscript. If an undisclosed competing interest comes to light after publication, Frontiers will take action in accordance with internal policies and Committee on Publication Ethics guidelines.

### D.3.5. Bioethics

All research submitted to Frontiers for consideration must have been conducted in accordance with Frontiers guidelines on study ethics. In accordance with COPE guidelines, Frontiers reserves the right to reject any manuscript that editors believe does not uphold high ethical standards, even if authors have obtained ethical approval or if ethical approval is not required.

#### D.3.5.1. Studies involving animal subjects

All research involving regulated animals (i.e. all live vertebrates and higher invertebrates) must be performed in accordance with relevant institutional and national guidelines and regulations. Frontiers follows (<http://www.veteditors.org/consensus-author-guidelines-on-animal-ethics-and-welfare-for-editors>) for publication of studies including animal research. Approval of research involving regulated animals must be obtained from the relevant institutional review board or ethics committee prior to commencing the study. Confirmation of this approval is required upon

submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the study. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

*This study was carried out in accordance with the principles of the Basel Declaration and recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee].*

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. Studies involving privately owned animals should demonstrate the best practice veterinary care and confirm that informed consent has been granted by the owner/s, or the legal representative of the owner/s. Frontiers supports and encourages authors to follow the ARRIVE guidelines for the design, analysis and reporting of scientific research.

#### *Humane Endpoints*

All manuscripts describing studies where death is an endpoint will be subject to additional ethical considerations. Frontiers reserves the right to reject any manuscripts lacking in appropriate justification.

#### D.3.6. Corrections

Frontiers recognizes our responsibility to correct errors in previously published articles. If it is necessary to communicate important, scientifically relevant errors or missing information, and compelling evidence can be shown that a major claim of the original article was incorrect, a Correction should be submitted detailing the reason(s) for and location(s) of the change(s) needed using the below template. Corrections can be submitted if a small portion of an otherwise reliable publication proves to be misleading, e.g. an error in a figure that does not alter conclusions OR an error in statistical data not altering conclusions OR mislabelled figures OR wrong slide of microscopy provided, or if the author / contributor list is incorrect when a deserving author has been omitted or somebody who does not meet authorship criteria has been included. The contribution to the field statement should be used to clearly state the reason for the Correction. Please note, a correction is not intended to replace the original manuscript.

#### D.3.7. Retractions

As a member of the (<http://publicationethics.org>), Frontiers abides by their guidelines and recommendations in cases of potential retraction.

Frontiers also abides by two other key principles, as recommended by COPE:

- Retractions are not about punishing authors.
- Retraction statements should be public and linked to the original, retracted article.

While all potential retractions are subject to an internal investigation and will be judged on their own merits, Frontiers considers the following reasons as giving cause for concern and potential retraction:

- Clear evidence that findings are unreliable, either as a result of misconduct (e.g. data fabrication) or honest error (e.g. miscalculation or experimental error)
- Findings have previously been published elsewhere without proper attribution, permission or justification (i.e. cases of redundant publication)
- Major plagiarism
- The reporting of unethical research, the publication of an article that did not have the required ethics committee approval
- Legal issues pertaining to the content of the article e.g. libellous content
- Major authorship issues i.e. proven or strongly suspected cases of ghostwriting or sold ('gift') authorship
- Politically-motivated articles where objectivity is a serious concern
- The singling out of individuals or organizations for attack
- Faith issues (e.g. intelligent design)
- Papers that have made extraordinary claims without concomitant scientific or statistical evidence (e.g. pseudoscience)

Readers who would like to draw the editors' attention to published work that might require retraction should contact the authors of the article and write to the journal, making sure to include copies of all correspondence with authors.

Please find more details on our comments and complaints policy (<https://www.frontiersin.org/about/publishing-model>).

### D.3.8. Support and Ethical concerns

In our commitment to continuously improve our website, we welcome your feedback, questions and suggestions. Please visit our Help Center to find guidance on our platform or contact us at (<mailto:support@frontiersin.org>).

**ADDENDUM E**

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**LETTERS OF CONSENT TO SUBMIT MANUSCRIPT**



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25 November 2019

Dear examiner

**MSc Dissertation – C Pieters**

**Permission to include manuscripts for examination purposes**

*As study leader and senior corresponding author on the article presented in Chapter 3, first authored by Ms Carmen Pieters, I hereby approve that the concept manuscript listed below be included as part of the requirements of her MSc degree, and that this manuscript may be submitted for examination of Ms. Pieters's dissertation.*

*The article is as follows:*

Chapter 3

**Evaluating selected neuro-biological and behavioural properties of efavirenz in an inflammatory model of schizophrenia, and response to the antioxidant N-acetylcysteine.**

Sincerely,



Dr Marisa Möller-Wolmarans

Study leader  
North-West University, Potchefstroom, South Africa



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25 November 2019

Dear examiner

**MSc Dissertation – C Pieters**

**Permission to include manuscripts for examination purposes**

*As co-author on the article presented in Chapter 3, first authored by Ms Carmen Pieters, I hereby approve that the concept manuscript listed below be included as part of the requirements of her MSc degree, and that this manuscript may be submitted for examination of Ms. Pieters's dissertation.*

*The article is as follows:*

Chapter 3

**Evaluating selected neuro-biological and behavioural properties of efavirenz in an inflammatory model of schizophrenia, and response to the antioxidant N-acetylcysteine.**

Sincerely,

Prof. Brian H. Harvey

Co-study leader

North-West University, Potchefstroom, South Africa