Bio-behavioural evaluation of efavirenz and Δ^9-tetrahydrocannabinol alone and in combination in rats with respect to psychosis and anxiety

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This letter serves as a declaration that this dissertation is the original work of N. Muller (24111996) and that it will only be submitted to the North West University (NWU) in partial fulfilment of the requirements of the degree *Master of Science* in Pharmacology.

X

Nadia Muller

Preface

"…nogtans sal ek in die Here jubel, sal ek juig in God, my Redder."

Habakuk 3:18

***

"Put your HOPE in God"

Psalm 43:5

***

"Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world"

Albert Einstein

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"Success is not final, failure is not fatal; It is the courage to continue that counts"

Winston Churchill

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Aan God kom al die eer toe. Alles wat ek is en ooit sal wees, is net genade!

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Abstract

The highly active antiretroviral therapy, efavirenz (EFV), has been noted to occur as a constituent of a potent new designer drug cocktail called “Nyaope” or “Whoonga”, which is reported to have mind-altering and addictive-like properties. As an anti-retroviral medicine for treatment against human immunodeficiency virus type-1 (HIV-1) induced acquired immunodeficiency syndrome (AIDS), EFV has been noted to induce a number of neuropsychiatric side effects viz. dizziness, hallucinations, sleep disturbances, anxiety, depression, impaired concentration, aggression, paranoia and acute psychosis. These neuropsychiatric effects clearly suggest the involvement of the central nervous system, which could provide insight on the reported addictive and abuse profile of EFV. Allegedly EFV is mixed with common household items (e.g. vinegar, detergents, baby powder, and rat poison) and other illicit substances (e.g. marijuana (containing Δ^9-tetrahydrocannabinol (Δ^9-THC), methamphetamine and/or heroin). This multi-drug cocktail is then either smoked or injected to produce a somnolent, euphoric sensation. The abuse profile and addictive properties of “Nyaope” increases the financial burden on the health support systems and agencies, encourages criminal activity and promotes resistance to antiretroviral therapies. Few preclinical studies have sought to elaborate on the addictive profile of EFV. One study noted that the pre-dominate behavioural profile of EFV is similar to that of the hallucinogen, lysergic acid diethylamide (LSD), with EFV having weak partial agonist activity at the serotonin 5-HT_{2A} receptor subtype, while another study reported that EFV induces depression and anxiogenic behaviour. More recently, the addictive properties of EFV has been established in a preclinical study, which reported that sub-acute and sub-chronic exposure to EFV (5 mg/kg) produces significant rewarding effects in rodents together with associated monoamine alterations in reward pathways of the brain. However, the deeper underlying mechanisms of this response remain unknown.

The primary aim of this study was to investigate the effects of a rewarding dose of EFV (5 mg/kg), Δ^9-THC (0.75 mg/kg) and the combination of EFV + Δ^9-THC on social interactive behaviour, sensorimotor domains as well as anxiety-like behaviour in rodents. The study (NWU-00278-17-A5) used 72 male, adolescent Sprague-Dawley rats (150g – 180g) divided into four equal groups (n = 18 per group). The animals were bred and housed under identical conditions in the Vivarium of the North-West University (NWU). The rats received alternate day drug-vehicle exposure to intraperitoneal injections of vehicle (pharmaceutical grade olive oil), EFV (5 mg/kg), Δ^9-THC (0.75 mg/kg) or the combination of EFV + Δ^9-THC (5 mg/kg and 0.75 mg/kg) for 17 days. The rats were subjected to well-validated behavioural tests to assess social interaction, anxiety and sensorimotor gating using the social interaction test (SIT), elevated plus maze (EPM) and pre-

pulse inhibition (PPI) of startle test, respectively. The SIT was specifically used to measure alterations in self-directed and social interactive behaviour.

The study secondary aimed to simultaneously investigate whether alterations in the above-mentioned behaviours could be attributed to neuroendocrine and immune-inflammatory alterations. Consequently, alterations in hippocampal oxytocin (OT) levels as well as alterations in plasma pro- and anti-inflammatory cytokines, viz. tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) respectively, were measured using enzyme-linked immunosorbent assay (ELISA) kits. Moreover, plasma tryptophan and its kynurenine metabolites (kynurenine, kynurenic acid (KYNA) and quinolinic acid (QA)) were determined using high-performance liquid chromatography (HPLC). One-way and two-way analysis of variance (ANOVA) and Bonferroni post hoc testing with multiple comparisons were used for statistical analysis, with $p < 0.05$ deemed statistically significant.

The results indicate that EFV induces deficits in social interaction, anxiogenic and psychotogenic behaviour when compared to the vehicle control. Δ^9-THC similarly induced alterations in social interaction and psychotogenic behaviour, but in contrast with EFV induced anxiolytic behaviour when compared to the vehicle control. The combination of EFV + Δ^9-THC promoted social behaviour and profound psychotogenic-like behaviour when compared to the vehicle control. Only the combination of EFV + Δ^9-THC was found to increase hippocampal OT concentrations compared to the control group, which paralleled the effects of this combination to increase social interaction behaviour compared to the control group. Exposure to EFV and Δ^9-THC alone induced a pro-inflammatory state by increasing plasma pro-inflammatory (TNF-α) and decreasing anti-inflammatory (IL-10) cytokines levels, respectively. Moreover, EFV, Δ^9-THC and the combination of EFV + Δ^9-THC induced significant disturbances in tryptophan metabolism as indicated by increased plasma levels of neurodegenerative QA and decreased plasma levels of KYNA, resulting in a reduced neuroprotective ratio (KYNA : Kynurenine).

This animal study provided insight on the bio-behavioural profile evoked by the use of EFV alone or in combination with Δ^9-THC, which may resemble that of “Nyaope”-cocktail users. Furthermore, the study has provided insight into possible neuroendocrine and peripheral immune-inflammatory mechanisms through which EFV induces its psychological behavioural profile.

Keywords: efavirenz, “Nyaope”, social interaction, anxiety, sensorimotor gating, oxytocin, cytokines, tryptophan.

Opsomming

Daar is opgemerk dat die hoogs-aktiewe antiretrovirale terapie, efavirenz (EFV), ‘n bestanddeel in die kragtige nuwe ontwerper-dwelm mengsel genaamd "Nyaope" of "Whoonga" is, wat na bewering geestesversteurings veroorsaak en verslawende eienskappe het. As anti-retrovirale terapie vir die behandeling van menslike immuniteitsgebreksvirus tipe 1 (MIV-1) geïnduseerde verworwe immuniteitsgebreksindroom (VIGS), is EFV in staat om 'n aantal neuropsigiatriese newe-effekte nl. duiseligheid, hallusinasies, slaapsteurnisse, angs, depressie, verswakte konsentrasie, aggressie, paranoia en akute psigose te veroorsaak. Hierdie neuropsigiatriese effekte dui duidelik op die betrokkenheid van die sentrale senuweestelsel en navorsing hieroor kan duidelikheid verskaf oor die gerapporteerde verslawings- en misbruiksprofiel van EFV. Daar word beweer dat EFV gemeng word met gewone huishoudelike items (bv. Asyn, skoonmaakmiddels, baba poeier en rotgif) en ander onwettige stowwe (bv. Marihuana (wat Δ^9-tetrahydrocannabinol (Δ^9-THC) bevat), metamfetamien en / of heroïen). Hierdie multi-geneesmiddel mengsel word dan gerook of ingespuit om 'n euforiese sensasie te produseer. Die misbruiksprofiel en verslawende eienskappe van “Nyaope” verhoog die finansiële las op die gesondheidsorgstelsels en -owerhede, moedig kriminele aktiwiteite aan en bevorder weerstand teen antiretrovirale terapie. Min pre-kliniese studies het al beoog om op die verslawingsprofiel van EFV na te vors. Een studie het opgemerk dat die pre-dominante gedragsprofiel van EFV soortgelyk is aan dié van die hallusinogeen, lysergiese suur-dietielamied (LSD), aangesien EFV ook oor swak gedeeltelike agonistiese aktiwiteit by die serotonien 5-HT_{2A}-reseptorsubtipe beskik, terwyl 'n ander studie berig het dat EFV depressie en angsverwekkende gedrag veroorsaak. Die verslawingspotensiaal van EFV is onlangs in ‘n pre-kliniese studie vasgestel nadat sub-akute en sub-chroniese blootstelling aan EFV (5 mg/kg) beduidende belonings-effekte in rotte gelewer het, tesame met gepaardgaande monoamienveranderinge in die belonings-baanweë van die brein. Die dieper, onderliggende meganismes van hierdie reaksies is egter nog nie bekend nie.

Die primêre doel van hierdie studie was om die effekte van 'n beloonende dosis EFV (5 mg/kg), Δ^9-THC (0.75 mg/kg) en die kombinasie van EFV + Δ^9-THC op sosiale interaktiewe gedrag, sensorimotoriese domeine en angsagtige gedrag in rotte te ondersoek. Die studie (NWU-00278-17-A5) het 72 manlike, adolessente Sprague-Dawley-rotte (150g - 180g) gebruik wat in vier gelyke groepe verdeel is (n = 18 per groep). Die diere was geteel en gehuisves onder identiese toestande in die Vivarium van die Noordwes-Universiteit (NWU). Die rotte het alternatiewe dagblootstelling van intraperitoneale inspuitings met die geneesmiddel-dragstof (farmaseutiese graad olyfolie), EFV (5 mg/kg), Δ^9-THC (0.75 mg/kg) of die kombinasie van EFV + Δ^9-THC (5 mg/kg en 0,75 mg/kg) vir 17 dae ontvang. Die rotte was blootgestel aan goed-gevalideerde

gedragstoetse om sosiale interaksie, angs en sensorimotoriese prosessering te assesseer deur onderskeidelik gebruik te maak van die sosiale interaksietoets (SIT), die verhewe-plusvormdoolhoftoets (VPT) en pre-puls inhibisie (PPI). Die SIT is spesifiek gebruik om tekortkominge in selfgerigte en sosiale interaktiewe gedrag te meet.

Die studie se sekondêre doel was om terselfdetyd te ondersoek of veranderings in bogenoemde gedrag toegeskryf kan word aan neuro-endokriene en immuun-inflammatoriese veranderinge. Gevolglik was enige veranderinge in hippokampale oksitosien (OT) vlakke asook veranderinge in plasma pro- en anti-inflammatoriese sitokiene (nl. tumor nekrosefaktor alfa (TNF-α) en interleukien-10 (IL-10) onderskeidelik), met behulp van ensiem gekoppelde immuun metodes (ELISA) gemeet. Daarbenewens was plasma triptofaan en sy kynurenien metaboliete (kynurenien, kynuriensuur (KS) en quinoliensuur (QS)) bepaal deur gebruik te maak van hoë-doeltreffendheid vloeistofchromatografie (HDVC). Een-rigting en twee-rigting analise van variansie (ANVVA) en Bonferroni post-hoc toetse met veelvuldige vergelykings was gebruik vir statistiese analises en ‘n $p < 0.05$ was as statisties betekenisvol geag.

Die resultate dui daarop dat EFV tekortkominge in sosiale interaksie induseer asook angswekkende en psigotiese gedrag veroorsaak wanneer dit met die kontrole groep vergelyk word. Δ^9-THC het psigotiese gedrag en soortgelyke tekortkominge in sosiale interaksie veroorsaak, maar in teenstelling met EFV het dit angswerende gedrag veroorsaak wanneer dit met die kontrole groep vergelyk word. Die kombinasie van EFV + Δ^9-THC het sosiale en psigotiese gedrag bevorder in vergelyking met die kontrole groep. Slegs die kombinasie van EFV + Δ^9-THC het ‘n toenmae in hippocampale OT konsentrasies veroorsaak in vergelyking met die kontrole groep. Hierdie toemane in hippokampale OT konsentrasies is in ooreenstemming met die toename in sosiale interaktiewe gedrag wat die kombinasie van EFV + Δ^9-THC in vergelyking met die kontrole groep veroorsaak het. Blootstelling aan EFV en Δ^9-THC alleen het 'n pro-inflammatoriese toestand veroorsaak deur onderskeidelik plasma pro-inflammatoriese sitokienvlakke (TNF-α) te verhoog en plasma anti-inflammatoriese sitokienvlakke (IL-10) te verlaag. Daarbenewens het EFV, Δ^9-THC en die kombinasie van EFV + Δ^9-THC beduidende versteurings in triptofaanmetabolisme veroorsaak, soos aangedui deur verhoogde plasmavlakke van neurodegeneratiewe QS en verlaagde plasmavlakke van KS, wat lei tot ‘n verlaging in die neuro-beskermende balans (KS: Kynurenien).

Hierdie dierstudie verskaf inligting rakende die bio-gedragsprofiel wat deur die misbruik van EFV alleen of in kombinasie met Δ^9-THC geproduseer word. Die bogenoemde bio-gedragsprofiel is moontlik soortgelyk aan dié van “Nyaope”-verbruikers. Verder verskaf hierdie studie insig oor

moontlike neuro-endokriene en perifere immuun-inflammatoriese meganismes waardeur EFV sy psigo-gedragsprofiel induseer.

Sleutelwoorde: efavirenz, “Nyaope”, sosiale interaksie, angs, sensorimotoriese prosessering, oksitosien, sitokiene, triptofaan.

CONGRESS CONTRIBUTIONS

Abstract for basic pharmacology podium presentation at the South African Society for Basic and Clinical Pharmacology congress, 7-10 October 2018, Spier Conference Centre, Stellenbosch, and Cape Town, South Africa. The student, as first and presenting author, won the 3rd prize in the "Basic Pharmacology" category.

Title:

Psychotogenic, anxiogenic and immune-inflammatory alterations in rats exposed to efavirenz alone or in combination with Δ^9-tetrahydrocannabinol

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Introduction: The recreational use of efavirenz (EFV) in a cannabis-containing cocktail commonly known as "Nyaope" has been described in South Africa. The dose-dependent rewarding effects of EFV (effective dose: 5 mg/kg) in rats has been established in our laboratory. Neuropsychiatric adverse events (NPAE) with EFV are common, particularly anxiety, social withdrawal, sensorimotor deficits and immune-inflammatory disturbances. The objective of this study was to establish the psychotogenic, anxiogenic as well as pro-inflammatory effects of EFV in rats alone and in combination with a known drug of abuse, Δ^9-Tetrahydrocannabinol (Δ^9-THC), compared to vehicle or Δ^9-THC alone.

Methods: 72 adolescent, male Sprague-Dawley rats (12 per group) were exposed to vehicle, EFV, Δ^9-THC or EFV+Δ^9-THC for 17 days (i.p. injections), alternating between drug- and vehicle exposure. Rats were subjected to the social interaction test (SIT), the elevated plus maze (EPM) and pre-pulse inhibition (PPI) test on day 12, 14 and 16 of exposure, respectively. Pro- (tumour necrosis factor alpha (TNFα)) and anti- (interleukin-10 (IL-10)) inflammatory cytokine levels were determined in plasma using sandwich enzyme-linked immunosorbent assay kits. (Ethics approval: NWU-00278-17-A5).

Results: EFV alone increased anxiety and self-grooming, induced deficits in social interaction and PPI, as well as increased plasma TNFα, but without affecting IL-10. Δ^9-THC alone increased self-grooming behaviour but decreased anxiety-like behaviour as well as decreased IL-10 levels

but without affecting TNFα. Combined EFV+Δ^9-THC exposure presented with decreased locomotor activity and %PPI, but increased social interactive behaviour withoutaltering cytokine levels.

Conclusion: The findings indicate that EFV exposure alone induces a pro-inflammatory state together with psychotogenic- and anxiogenic behavioural effects, not observed in rats exposed to Δ^9-THC alone. The combination of EFC+Δ^9-THC only presented with psychotogenic effects, while evidence would suggest that Δ^9-THC could possibly reverse other EFV-related NPAE.

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It is hereby certified that the

Young Scientist Award in Basic Pharmacology —3rd prize Podium Presentation

was awarded to

Nadia Muller

at the First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST 2018)

President: SASBCP

Vice-president: SASBCP

Chair: CoBNeST

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LIST OF ABBREVIATIONS

A

AA	Anthranilic acid
AC	Anterior cingulate
AIDS	Acquired Immune Deficiency Syndrome
AMG	Amygdala
AN	Accessory nuclei
ART	Antiretroviral therapy

B

BBB	Blood brain barrier
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis

C

cART	Combined antiretroviral therapy
CB	Cannabinoid

CB_1	Cannabinoid receptor subtype 1
CB_2	Cannabinoid receptor subtype 2
CNS	Central nervous system
CPP	Conditioned place preference
CRF	Corticotropin-releasing factor
CSF	Cerebrospinal fluid
CYP2B6	Cytochrome 2B6
CYP3A	Cytochrome 3A
CYP450	Cytochrome P450

D

D_1	Dopamine receptor subtype 1
D_2	Dopamine receptor subtype 2
DA	Dopamine
DAT	Dopamine transporters
DGP	Dorsal globus pallidus
DNA	Deoxyribonucleic acid

DOPAC	Dihydroxyphenylacetic acid
DS	Dorsal striatum

E

EFV	Efavirenz
EPM	Elevated plus maze

G

GABA	Gamma-aminobutyric acid
$GABA_A$	Gamma-aminobutyric acid receptor subtype A
GHB	Gamma-hydroxybutyrate
GP	Globus pallidus
GSH	Glutathione
GSSG	Glutathione disulfide

H

HAART	Highly active antiretroviral therapy

Hippo	Hippocampus
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HPA axis	Hypothalamic-pituitary-adrenal axis
HRP	Avidin-horseradish peroxidase
HVA	Homovanillic acid

I

i.p.	Intraperitoneal
IDO	Indoleamine-2,3-dioxygenase
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-13	Interleukin-13
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6

K

KAT	Kynurenine-aminotransferases
KMO	Kynurenine-3-monooxygenase
KYNA	Kynurenic acid

L

LHb	Lateral habenula
LSD	Lysergic acid diethylamide

M

MA	Methamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MFB	Medial forebrain bundle
MOB	Main olfactory bulb
mPFC	Medial prefrontal cortex
MPOA	Medial preoptic area
mRNA	Messenger ribonucleic acid

N

NAC	N-acetyl cysteine
NAcc	Nucleus accumbens
NE	Noradrenaline
NMDA	*N*-methyl-*D*-aspartate
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NO	Nitric oxide
NRTI	Nucleoside reverse transcriptase inhibitor

O

OFC	Orbitofrontal cortex
OT	Oxytocin

P

PFC	Prefrontal cortex
PI	Protease inhibitor
PND	Post-natal day

PPI	Pre-pulse inhibition
PVN	Paraventricular nucleus

Q

QA	Quinolinic acid

R

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcriptase

S

SD	Sprague-Dawley
SIT	Social interaction test
SNc	Substantia nigra pars compacta
SOD	Superoxide dismutase
SON	Supraoptic nucleus

T

TDO	Tryptophan-2,3-dioxygenase
Thal	Thalamus
TNF-α	Tumor necrosis factor alpha

U

UGT	UDP-glucuronosyltransferase
UNAIDS	Joint United Nations Programme on HIV/AIDS

V

VGP	Ventral globus pallidus
VMAT2	Vesicular monoamine transporter-2
VS	Ventral striatum
VTA	Ventral tegmental area

W

WHO	World Health Organization

Numbers

5-HT	Serotonin
5-HT_{1A}	Serotonin receptor subtype 1A
5-HT_{2A}	Serotonin receptor subtype 2A
5-HT_{2C}	Serotonin receptor subtype 2C
Δ^9-THC	Delta-9-tetrahydrocannabinol
8-OH-EFV	8-Hydroxyefavirenz

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CHAPTER 1

INTRODUCTION

This introductory chapter provides a brief overview of the study as a whole by focusing on the dissertation layout, problem statement (including a brief review on relevant recent literature and elaborated on in Chapter 2), study questions, study aims, hypothesis, project design, expected outcomes and ethical considerations.

1.1 Dissertation Approach and Layout

This dissertation is presented in article format as follows:

- **Chapter 1**
  - Problem statement, study questions and aims, hypothesis, study layout, expected outcomes and ethical considerations
- **Chapter 2**
  - Literature review
- **Chapter 3**
  - Article
    - Literature review
    - Materials and methods
    - Results
    - Discussion
- **Chapter 4**
  - Summary, conclusion and recommendations

1.2 Problem statement

Acquired Immune Deficiency Syndrome (AIDS) is regarded as one of the most devastating pandemics internationally (Sued *et al.*, 2016) and is caused by a retrovirus termed the human immunodeficiency virus (HIV) (Dubey *et al.*, 2017). HIV is responsible for progressive immunosuppression by reducing the $CD4^+$ T-cell counts and infecting macrophages (Sued *et al.*, 2016; Dubey *et al.*, 2017). According to the most recent fact sheet (June, 2017) of the Joint United Nations Programme on HIV/AIDS (UNAIDS) (UNAIDS, 2017), a global average of 1.8 million people became newly infected with HIV in 2016. An overwhelming 43% of these new HIV infections worldwide occurred only in eastern and southern Africa, making sub-Saharan Africa the most affected continent (Piot & Quinn, 2013). These statistics emphasize the importance and need for highly active antiretroviral therapy (HAART). HAART supresses the HIV viral load and strengthens the immune system (Bartlett & Gallant, 2000) and consists of several combinations of antiretroviral drugs (Raines *et al.*, 2005). The World Health Organization's most recent guidelines suggests that efavirenz (EFV) is the preferred NNRTI for HAART (Organization, 2016), most likely due to its superior virological efficacy (Arribas, 2003). However, EFV is the antiretroviral therapy (ART) associated with the most central nervous system (CNS) and neuropsychiatric adverse effects including hallucinations, abnormal and vivid dreams, acute psychosis, aggression, paranoia, anxiety and depression (Marzolini *et al.*, 2001; Lochet *et al.*, 2003; Raines *et al.*, 2005; Gatch *et al.*, 2013). The psychological effects caused by EFV indicate clear CNS involvement, which could lead to potential abuse and even drug-addiction.

In fact, several reports have described the recreational use of EFV among South African adolescents [as young as 13 years old (Mokwena & Huma, 2014)] for its psychological effects (Sciutto, 2009; Cullinan, 2011), especially in townships and other rural areas (Mokwena & Huma, 2014). EFV tablets (approximately 1 tablet equivalent to 600 mg EFV) are crushed and mixed with substances such as heroin, Δ^9-tetrahydrocannabinol (Δ^9-THC) (the psychological component in marijuana), rat poison, detergents, vinegar and baby powder to produce a potent multi-drug cocktail called "Nyaope" or "Whoonga" (Hull, 2010; Cullinan, 2011; Fihlani, 2011; Mokwena & Huma, 2014). This cocktail is then either smoked or injected intravenously to produce the reported sensation of 'getting high' (Marwaha, 2008; Tshipe, 2017). "Nyaope"-users reported feeling relaxed, somnolent and euphoric after smoking it, potentially leading to drug-dependency and drug-cravings, the latter generally associated with physical pain and discomfort (Marwaha, 2008; Mokwena & Huma, 2014). "Nyaope" is readily and easily available and is a relatively inexpensive recreational drug, ranging between R20-R30 per consumable quantity ('hit' or 'fix') (Fihlani, 2011; Mokwena & Huma, 2014).

EFV abuse limits the roll-out of anti-HIV medicines which in turn increases the financial burden on the Department of Health. Moreover, many HIV-negative "Nyaope"-addicts actively seek infection with HIV in order to receive free ART (which they will then use recreationally) (Hull, 2010). For certain addicts, becoming involved in criminal activity is the only way to secure their daily "Nyaope"-supply, whereupon health care facilities and HIV-positive patients are being targeted by desperate "Nyaope"-addicts. Several HIV-positive patients reported that they feel unsafe when collecting their HIV-medication as they fear that they might be robbed or mugged of their life-saving ART (Fihlani, 2011). This acts as a deterrent preventing them from collecting their medication or even seeking future treatment (Fihlani, 2011).

Even more problematic is that treatment-naïve HIV-positive individuals may become resistant to ART when using EFV recreationally (Grelotti *et al.*, 2013), due to the formation of NNRTI resistant mutations (Kasang *et al.*, 2011). Resistance to ART will typically result in future treatment-failure which will adversely affect the international HIV treatment-response (Gupta *et al.*, 2012).

Research is needed in order to determine the underlying neuro-pharmacological mechanisms and behavioural effects of EFV abuse, and devising strategies how it may be treated. This information may also assist in countering such practice in the first place. Currently, there is also very little literature documenting the effects of the recreational use of EFV compared to other known drugs of abuse.

Earlier studies have established the importance of serotonin (5-HT) transmission in the neurological effects of EFV (Gatch *et al.*, 2013), as well as its depressogenic and anxiogenic potential (Cavalcante *et al.*, 2017). Recently our laboratory also established that sub-chronic exposure to the most rewarding dose of EFV (5 mg/kg; established in the condition place preference (CPP) paradigm) increases cortico-striatal dopamine (DA) and 5-HT levels as well as striatal noradrenaline (NE) concentrations. Furthermore, sub-chronic exposure to EFV (5 mg/kg) also produced altered redox states with increased production of reactive oxygen species (ROS), lipid peroxidation and peripheral oxidative stress. However, detail on the involvement of immune-inflammatory alterations, as well as their relationship with anxiety, altered social behaviour and sensorimotor domains linked to EFV exposure has not been addressed. The latter is seen as important, given that these pathways are closely related to neurotoxicity and subsequent neurodegeneration, which could possibly underlie the bio-behavioural responses to other known drugs of abuse. Previous work has linked disturbances in kynurenine metabolism to psychiatric illnesses such as depression (Myint *et al.*, 2012), psychosis and schizophrenia (Muller *et al.*, 2011; Erhardt *et al.*, 2017). It has been widely established that drugs of abuse such as methamphetamine (MA) and psychostimulants cause neurotoxicity by inducing a pro-

inflammatory state in the brain (Thomas *et al.*, 2004; Cadet *et al.*, 2007; Clark *et al.*, 2013). This pro-inflammatory state can be produced by several mechanisms, including: increases in pro-inflammatory cytokines (such as tumor necrosis factor alpha (TNF-α)), decreases in anti-inflammatory cytokines (such as Interleukin-10 (IL-10)) and the production of oxidative stress (Möller *et al.*, 2015). The pro-inflammatory state will then shift tryptophan metabolism via the kynurenine pathway by activating tryptophan-2,3-dioxygenase (TDO) or indoleamine-2,3-dioxygenase (IDO) (Möller *et al.*, 2015) which metabolizes tryptophan into kynurenine. Kynurenine will be metabolised to either the neuroprotective *N*-methyl-*D*-aspartate (NMDA) receptor antagonist, kynurenic acid (KYNA) (Connor *et al.*, 2008; Han *et al.*, 2010) or quinolinic acid (QA), a NMDA receptor agonist and excitotoxin with neurodegenerative properties (Schwarcz, 2004; Connor *et al.*, 2008; Möller *et al.*, 2015).

The investigation into the long-term neurotoxic effects (if any) induced by sub-chronic EFV-abuse warrants further research. The use of well-validated behavioural tests as applied in the current study will be of particular value to determine whether EFV abuse can be linked to deficits in social interactive behaviour (social interaction test (SIT)), increased anxiety (elevated plus maze (EPM)) and psychosis-like behaviour (prepulse inhibition (PPI) test). It is important to consider that the effects of drugs of abuse on social interactive behaviour are complex and could potentially involve interactions with the hypothalamic peptide hormone, oxytocin (OT), which has been implicated in 'pro-social' and anxiolytic behaviour (McRae-Clark *et al.*, 2013). Drugs of abuse such as 3,4-methylenedioxymethamphetamine (MDMA) is noted for promoting social interaction between unfamiliar rat pairs (Morley & McGregor, 2000; Thompson *et al.*, 2007), an effect most likely produced by MDMA-induced increases in OT-release (Thompson *et al.*, 2007). Thus, the evaluation of monoamine-induced alterations and oxidative-inflammatory responses (performed by Fourie *et al.* (2017)) can provide a basis for the aforementioned behaviours that could help in explaining the reported abuse potential of EFV, its possible neurodegenerative properties, and reveal more on how it may be treated and/or prevented.

1.3 Study questions

Based on the above-mentioned problem identification, the study questions are:

1. Which behavioural and immune-inflammatory alterations result from sub-chronic exposure to EFV in rats (if any)?

2. How do any of the above-mentioned EFV-induced bio-behavioural alterations compare with that of Δ^9-THC, a known drug of abuse?
3. Is the combination of EFV and Δ^9-THC synergistic with regards to any of the above-mentioned bio-behavioural effects?

1.4 Study aims

We identified the following project aims in order to successfully answer the above-mentioned study questions:

1.4.1 Primary aims

- To investigate the effects of EFV, Δ^9-THC as well as the combination of EFV + Δ^9-THC exposure in rats on social interactive behaviour (utilizing the SIT), anxiety-like behaviour (utilizing the EPM) and sensorimotor gating (utilizing PPI of the acoustic startle reflex), compared to rats only receiving vehicle (pharmaceutical grade olive oil).
- To compare the respective exposure groups with each other in order to observe a possible trend in the behavioural profile induced by each drug and to establish whether the drugs act synergistically with one another.

1.4.2 Secondary aims

- To investigate whether altered behaviours (if any) in EFV, Δ^9-THC and EFV + Δ^9-THC exposed rats are associated with altered oxytocin (OT)- and plasma pro- and anti-inflammatory cytokine levels as well as altered tryptophan-kynurenine metabolism compared to rats only exposed to vehicle.
- To compare the neuroendocrine and immune-inflammatory alterations induced by EFV, Δ^9-THC and EFV + Δ^9-THC exposure with each other in order to observe any trends in the mechanisms by which these drugs possible produce behavioural alterations.

1.5 Hypothesis

Rats subjected to sub-chronic EFV exposure will present with increased anxiety and significant deficits in social interactive behaviour (such as social withdrawal) and sensorimotor gating. These behavioural changes will be associated with decreased hippocampal OT levels and increased plasma levels of TNF-α (pro-inflammatory cytokine) and QA, as well as decreased plasma levels of IL-10 (anti-inflammatory cytokine) and KYNA. These bio-behavioural alterations will be more

pronounced with the addition of Δ^9-THC (EFV + Δ^9-THC) compared to rats only receiving EFV. EFV-induced bio-behavioural alterations will also be comparable to alterations induced by Δ^9-THC, a known drug of abuse.

1.6 Study layout

Figure 1-1 depicts the layout of the study. 72 male Sprague-Dawley (SD) rats were randomly divided into four main groups (of equal size; n = 18 per group) by an experienced animal technologist blind to the study, this in order to remove bias from the experimental design (ARRIVE guidelines) (Kilkenny *et al.*, 2010). The rats received alternate day dosing of either vehicle (pharmaceutical grade olive oil) intraperitoneally (i.p.), EFV (5 mg/kg/day i.p.) (Möller *et al.*, 2018), Δ^9-THC (0.75 mg/kg/day i.p.) (Braida *et al.*, 2004) or EFV + Δ^9-THC (respective dosages mentioned above), for a period of 17 days (from post-natal day (PND) +49 to PND +65). The rats were then subjected to sequential behavioural tests, viz. SIT on day 12 (PND +60), EPM on day 14 (PND +62) and PPI of the acoustic startle reflex on day 16 (PND +64). To ensure cost-effectiveness and compliance with ethical standards (specifically the reduction principle), the same rats were used to assess behavioural as well as neurochemical alterations. The rats were euthanized (by decapitation) on day 17 of drug exposure (PND +65), 2 hours after receiving the last drug dose, in order to prevent a drug-withdrawal effect on the neurochemical analysis. This wash-out period was sufficient, due to the short elimination half-life of EFV in rats which ranges from 0.8 to 1.9 hours compared to 40 + hours in humans (Anon., 2007). After euthanasia, the hippocampus was dissected for determination of OT levels with trunk blood collected for inflammatory cytokine and tryptophan-kynurenine pathway analysis.

In order to avoid investigator bias, all experiments and analyses were performed by an investigator blind to the exposure conditions, this in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010).

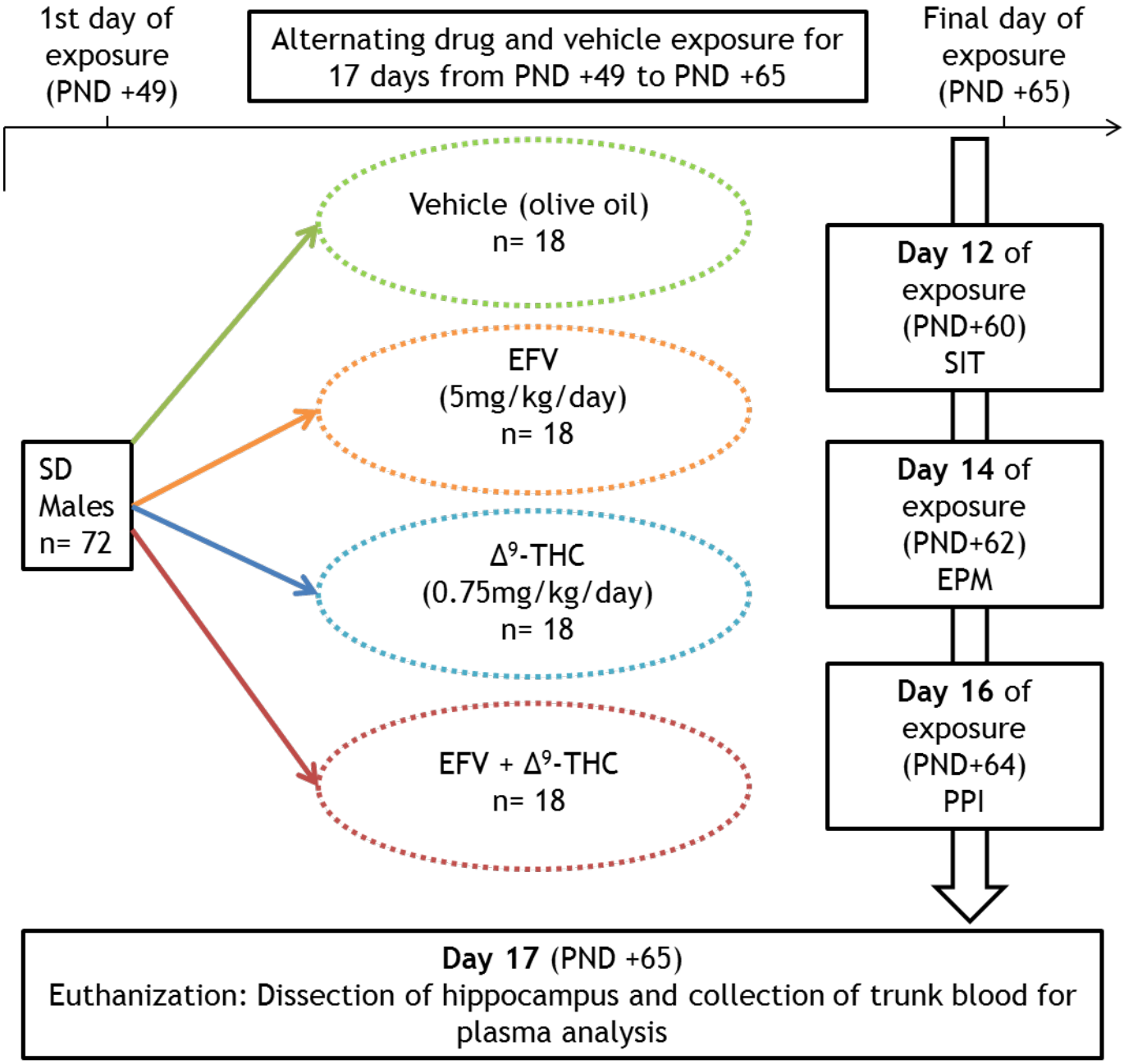

Figure 1-1: Experimental design of the study. Throughout this sub-chronic study a total of 72 male SD rats received alternate day dosing of either a vehicle (pharmaceutical grade olive oil) or EFV (5mg/kg/day i.p.) or Δ^9-THC (0.75mg/kg/day i.p.) or both EFV and Δ^9-THC (respective dosages mentioned) for a total of 17 days from PND +49 to PND +65. The rats were then subjected to the SIT, EPM and PPI behavioural tests on PND +60, PND +62 and PND +64 respectively; thereafter they were euthanized, 24 hours after the last behavioural test and 2 hours after they received their last drug dose (on PND +65). SD= Sprague-Dawley; PND= Post-natal day; EFV= Efavirenz; Δ^9-THC= Δ^9-Tetrahydrocannabinol; SIT= Social interaction test; EPM= Elevated plus maze; PPI= Prepulse inhibition; PFC= Prefrontal cortex.

1.7 Expected outcomes

We proposed the following outcomes:

- Rats exposed to sub-chronic EFV will exhibit decreased social interactive behaviour (by spending less time engaging in social interaction) and increased self-directed social behaviour (by spending more time engaging in non-social self-exploratory activity viz. self-grooming) in comparison to the control group.

- Sub-chronic EFV exposure will increase anxiety-like behaviour (in the EPM) in comparison to the control group.

- Sub-chronic exposure to EFV will reduce the % PPI, indicative of deficits in sensorimotor gating in comparison to the control group.

- Sub-chronic EFV will induce the following neurochemical and plasma alterations in comparison to the control group:

  - Decreased hippocampal OT levels.

  - Increased plasma levels of the pro-inflammatory cytokine, TNF-α, and decreased plasma levels of the anti-inflammatory cytokine, IL-10.

  - Increased pro-inflammatory response which will shift tryptophan metabolism towards the formation of kynurenine, resulting in increased levels of the neurodegenerative QA and decreased levels of neuroprotective KYNA and a decrease in the neuroprotective ratio (KYNA: kynurenine).

- The above-mentioned bio-behavioural and neurochemical alterations will be similar or less severe in rats only exposed to Δ^9-THC, whereas rats exposed to EFV + Δ^9-THC will present with more pronounced alterations vs. either drug alone.

1.8 Ethical considerations

SD rats were bred and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform of the North-West University (NWU). The study was approved by the AnimCare animal research committee

(NHREC reg. no. AREC-130913-015) of the NWU. Animal handling, injection procedures and behavioural testing were carried out in accordance with the code of ethics in research, training and drug-testing in South Africa and complied with national legislation (Ethics approval number: NWU-00278-17-A5).

The 4 R's of ethical research have been addressed as follows:

Replacement: The use of well-validated animal (rat) models during drug-addiction studies are preferred since both humans and rodents are susceptible to substance-addiction and share the same neurochemical reward pathway on which addictive substances will exert their effects (Kalivas *et al.*, 2006). Therefore, rats can be used to study the behavioural effects and neurochemical alterations induced by drugs of abuse.

Reduction: Animal numbers were based on the recognized number of 18 rats per group as outlined by similar studies (Toua *et al.*, 2010) after consultation from a statistician of the NWU.

Refinement: The researcher used study-specific NWU Vivarium monitoring sheets on a daily basis to evaluate the rats for any discomfort or excessive stress.

Responsibility: The researcher accepted full responsibility and oversaw each aspect of the entire study to ensure that the study was in line with scientific and ethical standards and guidelines.

Considering the risk : benefit ratio, this study will provide new evidence on the pharmacological, neurochemical and behavioural mechanisms underlying the neuropsychiatric manifestations related to the addictive profile and abuse properties of EFV in rats, with or without the addition of Δ^9-THC.

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CHAPTER 2

LITERATURE REVIEW

2.1 Human Immunodeficiency Virus and highly active antiretroviral therapy

Since the discovery of the potentially fatal, progressive immunosuppressing disease- Acquired Immune Deficiency Syndrome (AIDS) in 1981 (Greene, 2007; Sharp & Hahn, 2011), it has evolved to become one of the most cataclysmic global pandemics of the last century (Gallo *et al.*, 1984; Popovic *et al.*, 1984; Barre-Sinoussi *et al.*, 2004; Sued *et al.*, 2016). The causative agent is a retrovirus termed the human immunodeficiency virus (HIV) (Dubey *et al.*, 2017), more specifically the human immunodeficiency virus type 1 (HIV-1) (Cohen *et al.*, 2011). However, AIDS can also result from an infection with another lentivirus closely related to HIV-1 namely HIV type 2 (HIV-2) (Sharp & Hahn, 2011). Popper *et al.* (1999) stated that HIV-2 differs from HIV-1 in that it is less pathogenic, a conclusion based on the finding that plasma viremia in HIV-2 infection tends to be significantly lower than those in HIV-1 infected individuals. Rowland-Jones and Whittle (2007) further elaborated and found that the HIV-2 infection does not progress to AIDS in the majority of infected individuals. According to the most recent fact sheet (June, 2017) of the Joint United Nations Programme on HIV/AIDS (UNAIDS), a global average of 36.7 million people are infected with HIV, with approximately 1.8 million global new infections occurring only in 2016. An overwhelming 43% of these new HIV infections worldwide occurred only in eastern and southern Africa (UNAIDS, 2017), supporting Piot and Quinn (2013) finding that sub-Saharan Africa is the most affected continent. However the percentage new infections in eastern and southern Africa declined by 29% from 2010 to 2016 (UNAIDS, 2017). This dramatic decline can primarily be attributed to the government roll-out of antiretroviral medicines but also the efficacy of highly active antiretroviral therapy (HAART), which is considered to be the 'golden standard' for treating HIV positive individuals (Sendi *et al.*, 2001). HAART supresses the HIV viral load and strengthens the immune system by increasing the $CD4^+$ T-cell counts (Bartlett & Gallant, 2000), thereby reducing morbidity. HAART is also revolutionizing the HIV sector by converting AIDS from a mortal to chronic, but manageable, disease (Apostolova *et al.*, 2017). HAART and other antiretroviral therapy (ART) provide a platform for disease-management (Broder, 2010). From this platform we have been able to observe that although there was a significant decline in the prevalence of AIDS and HIV-related deaths after the introduction of HAART in 1996, people are still becoming infected with HIV (Febvey *et al.*, 2015). HAART typically consists of combinations of nucleoside reverse transcriptase inhibitors

(NRTIs) (such as zidovudine, didanosine, zalcitabine and stavudine), non-nucleoside reverse transcriptase inhibitors (NNRTIs) (including efavirenz (EFV), delavirdine and nevirapine), protease inhibitors (PIs) (such as indinavir, ritonavir or saquinavir) and fusion inhibitors (Raines *et al.*, 2005). EFV is currently employed by the World Health Organization (WHO) as first line HIV-1 treatment together with a non-thymidine NRTI combination (tenofovir disoproxil fumarate and emtricitabine or tenofovir disoproxil fumarate and lamivudine) (WHO, 2010).

2.2 Efavirenz

The superior virological efficacy of EFV has greatly contributed to its preferential, dominant profile in combined antiretroviral therapy (cART) (Sierra-Madero *et al.*, 2010; Dalwadi *et al.*, 2016). A previous clinical study found that a treatment regimen with EFV and two NRTIs is the most effective initial treatment for HIV-1 (Riddler *et al.*, 2008). Another recent study also described EFV as the NNRTI "*par excellence*" considering that it is regarded as first-line therapy for treatment-naïve, treatment-experienced and difficult-to-treat patients in combination with the following NRTIs: lamivudine / zidovudine in Combivir®, with abacavir / lamivudine in Kivexa®, or with emtricitabine / tenofovir disoproxil fumarate in Truvada® (Apostolova *et al.*, 2017).

2.2.1 Classification and mechanism of action

The benzoxamine compound, EFV [(4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one, has been commercially available since 1998 as Sustiva® or Stocrin®], and is a highly potent NNRTI (Arribas, 2003). EFV inhibits the catalytic production of proviral deoxyribonucleic acid (DNA) from viral ribonucleic acid (RNA) by diminishing HIV reverse transcriptase (RT) enzyme activity (Jacobo-Molina & Arnold, 1991; De Clercq, 2009; de Béthune, 2010). This phenomenon can be attributed to the ability of NNRTIs (such as EFV) to induce conformational changes by binding non-competitively to the spatial and functional hydrophobic pocket on the RT enzyme (Debyser *et al.*, 1992; Dueweke *et al.*, 1992; De Clercq, 2009; de Béthune, 2010) (as illustrated in figure 2-1) and thereby changing the position of crucial amino acids (Cohen *et al.*, 1991).

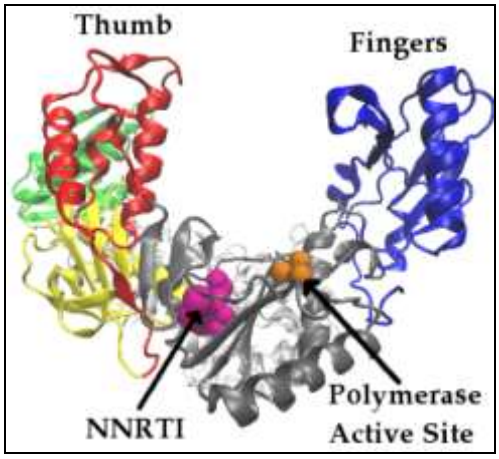


Figure 2-1: Schematic representation of the structure of the HIV-1 RT and the location of the bound NNRTI EFV (shown in magenta) (Wright *et al.*, 2012). *NNRTI, non-nucleoside reverse transcriptase inhibitor.*

2.2.2 Pharmacokinetics

A unique characteristic of EFV with regards to other NNRTIs is that it is approximately 99.8% bound to plasma-proteins (mainly albumin) (Tanaka *et al.*, 2008; Avery *et al.*, 2013a; Dalwadi *et al.*, 2016). However, it demonstrates sufficient penetration into the cerebrospinal fluid (CSF) usually reaching a concentration of 11.1-30 μg/L and can still be detected in the CSF 20 hours after administration due to its long plasma half-life (ranging from 40 to 60 hours in humans (Wynn *et al.*, 2002; Bastos *et al.*, 2016) and 0.8 to 1.9 hours in rats (Anon., 2007)). This extensive plasma half-life of EFV permits a single daily dose of 600 mg, which will typically result in a plasma concentration of 1-4 mg/L (Staszewski *et al.*, 1999). This plasma concentration of EFV is sufficient to ensure virologic success by suppressing the HIV-1 virus to clinically significant levels (Marzolini *et al.*, 2001).

EFV is considered to have sufficient central nervous system (CNS) penetration as it readily crosses the blood brain barrier (BBB) due to its lipophilic physiochemical properties (Abdissa *et al.*, 2015). A preclinical study in rats demonstrated that 15 mg/kg intraperitoneally injected EFV readily accumulated in the brain to levels that exceed 4.6 times the plasma levels (Dirson *et al.*, 2006). This is clinically important since the CNS is one of the primary sites for HIV replication (Wynn *et al.*, 2002).

EFV is primarily (90%) metabolized by the hepatic cytochrome P450 (CYP450) system and more specifically by the 2B6 (CYP2B6) isoenzyme with little involvement of cytochrome 3A (CYP3A) (Ward *et al.*, 2003). CYP2B6 is responsible for generating hydroxylated metabolites which are implicated to play a key role in EFV-associated CNS adverse effects (Ogburn *et al.*,

2010). The oxidized EFV metabolites are then conjugated by UDP-glucuronosyltransferase (UGT) during phase 2 of hepatic biotransformation (Apostolova *et al.*, 2017) and excreted in the urine (Adkins & Noble, 1998).

2.2.3 Side-effects

Despite the reported safe and tolerable pharmacological profile of EFV (Arribas, 2003; Best & Goicoechea, 2008), which generally leads to improved adherence and treatment outcomes (Arribas, 2003), EFV is still associated with significant side effects. These are mostly mild to moderate in severity and usually subside within a few weeks after the initiation of EFV therapy (Marzolini *et al.*, 2001)), and include hepatotoxicity, skin rash and CNS adverse effects.

EFV is the ART associated with the most neuropsychiatric adverse effects, as 73% of patients who initiated EFV-therapy experienced some form of CNS symptoms (Cespedes & Aberg, 2006). Some of the most concerning CNS and neuropsychiatric side effects include: dizziness, hallucinations, frequent nightmares, abnormal and vivid dreams, insomnia, anxiety, depression, impaired concentration and cognitive function, aggressive behaviour, paranoia, acute psychosis and even suicidal tendencies (Adkins & Noble, 1998; Marzolini *et al.*, 2001; Puzantian, 2002; Lochet *et al.*, 2003; Raines *et al.*, 2005; Gatch *et al.*, 2013). Some of these symptoms, which commonly peaks in the first 2 weeks after initiation of treatment, have been observed in patients on a treatment regimen of 600 mg EFV / day (Lowenhaupt *et al.*, 2007; Kenedi & Goforth, 2011). However, it should be mentioned that numerous clinical studies have indicated that CNS side effects may be a cause of slow hepatic metabolism and acute overdose (Hasse *et al.*, 2005; Lowenhaupt *et al.*, 2007). One clinical study specifically observed an association between CNS side effects and CYP2B6 (expressed in the liver, neurons and astrocytes) activity (Haas *et al.*, 2004). CYP2B6 is the isoenzyme primarily responsible for the hydroxylation of EFV to form 8-hydroxyefavirenz (8-OH-EFV) - the most important metabolite implicated in EFV-associated neuropsychiatric symptoms (Ogburn *et al.*, 2010), taking into account that 8-OH-EFV accumulates in both the plasma and CSF (Tovar-y-Romo *et al.*, 2012; Avery *et al.*, 2013b). A recent preclinical study defined 8-OH-EFV as a potent neurotoxin, considering that it induced significant, concentration-dependent damage to dendritic spines in a rat hippocampal neuron culture model (Tovar-y-Romo *et al.*, 2012). In another preclinical *in vitro* study, 8-OH-EFV notably altered glucose metabolism in astrocytes by increasing glucose consumption as well as the extracellular accumulation of lactate in a time- and dose-dependent, reversible manner (Brandmann *et al.*, 2013). The increase (mediated by 8-OH-EFV) in the glycolytic flux can drastically affect the performance of neurons due to close metabolic coupling of astrocytes (Hirrlinger & Dringen, 2010; Pellerin & Magistretti, 2012) and the risks associated with brain acidosis (Goldman *et al.*, 1989; Testai & Gorelick, 2010). Brandmann *et al.* (2013) also

concluded that apart from stimulating astrocytic glucose metabolism, 8-OH-EFV also inhibits mitochondrial respiration which will give rise to significant neuropsychiatric symptoms due to impaired neuronal energy production. The fact that 8-OH-EFV influences the glycolytic flux in cultured rat astrocytes (Brandmann *et al.*, 2013) is of clinical relevance since astrocytes are the most profuse cell type in the brain and are responsible for several vital functions, including extracellular ion homeostasis, neurotransmitter clearance, metabolite supply to other neurons and regulating synaptic transmission (Hirrlinger & Dringen, 2010; Parpura *et al.*, 2012; Dienel, 2013).

2.2.4 Abuse potential of efavirenz

The psychoactive effects caused by EFV indicate clear CNS involvement, which could lend credence to its possible abuse and addiction. In fact, since 2010 there were several news reports describing the recreational use of EFV as an apparent drug cocktail amongst South African adolescents, commonly known as “Nyaope” or “Whoonga” (Marwaha, 2008; Sciutto, 2009; Hull, 2010; Cullinan, 2011; Fihlani, 2011). “Nyaope” or “Whoonga” allegedly contains crushed EFV tablet/s (approximately 1 tablet or 600 mg) mixed with illicit substances such as marijuana, methamphetamine (MA) and/or heroin (Hull, 2010; Larkan *et al.*, 2010) as well as common household products including vinegar, detergents, baby powder and rat poison (Hull, 2010; Cullinan, 2011; Fihlani, 2011; Mokwena & Huma, 2014).

Moreover, a recent preclinical study observed that the predominate behavioural profile of EFV in rats is very similar to that of lysergic acid diethylamide (LSD), which was confirmed in radio ligand binding assays where EFV was found to act as a weak partial agonist at the serotonin 5-HT_{2A} receptor subtype (Gatch *et al.*, 2013). Cavalcante *et al.* (2017) also recently reported that sub-chronic EFV exposure induces anxiety (at a dose of 50 mg/kg) and depression-like behaviour (at 25 mg/kg and 50 mg/kg) in rats as well as increases in striatal glutamate and decreases in striatal monoamine (dopamine (DA), serotonin (5-HT) and noradrenaline (NE)) as well as gamma-aminobutyric acid (GABA) concentrations. Recent findings in our laboratory also indicate a dose-dependent rewarding effect of sub-chronic (14 days) EFV exposure in the conditioned place preference (CPP) paradigm, together with increased cortico-striatal levels of DA and 5-HT as well as increased NE levels in the striatum (Möller *et al.*, 2018). Moreover, sub-chronic EFV also produced profound alterations in redox states with increased lipid peroxidation and peripheral oxidative stress (Möller *et al.*, 2018).

2.3 Drug dependence, reward and addiction

Before reviewing the neurocircuitry of drug addiction and reward, one should consider the aetiology of addiction. In this regard, it is important to define and understand the terms 'reward' and 'drug addiction' and 'drug dependence'.

2.3.1 Reward

Although 'reward' has not been uniformly defined, in the broad sense of the word it can refer to any stimuli (environmental or circumstantial) that produce a positive effect on several conceptual aspects of behaviour, attitudes etc.. Therefore Ikemoto and Bonci (2014) operationally defined reward as "an induced state that subsequently leads to conditioned approach behaviour". Therefore the term 'reward' suggests subjective responses associated with the effects induced by illicit drug-intake (Everitt & Robbins, 2005).

The ventral tegmental area (VTA)-ventral striatum dopaminergic system, also known as the mesocorticolimbic dopaminergic system, is considered to be a common pathway by which drugs of abuse mediate their rewarding effects (Salamone, 1994; Berridge & Robinson, 1998; Ikemoto & Panksepp, 1999). The projection of dopaminergic neurons from the VTA to the ventral striatum plays an important role in mediating rewarding effects (Fibiger & Phillips, 1986; Koob, 1992). Moreover, Ikemoto and Bonci (2014) speculated that activation of this system alters sub-conscious processes followed by DA-mediated changes in the conscious mind, inducing positive changes in feelings and emotions. An example supporting this speculation is that administration of the psychostimulant, cocaine, produces euphoria due to the activation of the dopaminergic system (Ikemoto, 2007; Ikemoto, 2010). Therefore, it appears that DA plays an important role in reward, taking into account that drug-induced increases in DA can enhance drug-craving and sustain the continuous need to take the drug (Fiorentini *et al.*, 2010).

However, the implied definition of reward does not necessarily implicate that it is accompanied by a positive conscious experience mediated by increased DA release, that is if we consider that inhibition of DA receptors in the ventral striatum disrupts instrumental responding for sucrose solutions, but not the consumption thereof (Ikemoto & Panksepp, 1996). However, the increase in DA levels induced by drugs of abuse will contribute to compulsive drug-seeking behaviour and consequential addiction, regardless of whether the drug-induced effects are consciously perceived as pleasurable or not (Volkow & Li, 2004).

2.3.2 Drug addiction

Drug addiction can be defined as compulsive drug-seeking behaviour that results in chronic use of the drug concomitant with the inability to exercise control over the intense urges to take the

drug (therefore it is also characterized by multiple relapses (Yuan *et al.*, 2017)), despite the development of potential significant adverse effects and at the expense of other daily activities (Edwards *et al.*, 1981; Volkow & Li, 2004; Yuan *et al.*, 2017). Therefore, clinicians in addiction often refer to drug addiction as the disease with the 5 Cs:

> *"C*hronic disease with impaired *C*ontrol, *C*ompulsive use, *C*ontinued use despite harm, and *C*raving for the drug(s) to which the individual has become addicted".
>
> -(O'Brien & Gardner, 2005)

Obsessive drug-cravings and subsequent drug addiction mostly result from the effects (whether rewarding or aversive) derived from drugs of abuse. This refers to the ability to induce changes in the mental state or conscious experience of the user, to enhance physical and / or mental capabilities or to induce an euphoric state (Volkow & Li, 2004), and its complex interactions with environmental and biological factors (Volkow & Li, 2004).

Changes in certain biological factors such as normal neural development processes may be precursors of drug addiction (Volkow & Li, 2004). Thus, the risk of drug abuse and the potential development of drug addiction is higher during certain life stages and especially during adolescence, where brain regions responsible for executive control and motivation are still developing (Wagner & Anthony, 2002). Incomplete development of those particular brain regions will contribute to normal adolescent behaviour (such as the urge to experiment with novelties, to take risks and to fall under the burden of peer pressure) which generally provokes drug-experimentation (Spear, 2000).

As previously mentioned, the rewarding and reinforcing effects of drugs of abuse can be attributed to the activation of dopaminergic pathways in the striatum and hippocampus (Schultz *et al.*, 1998). Moreover, drug addiction results from neuroadaptations induced by chronic supraphysiological perturbations of the dopaminergic system (Volkow & Li, 2004). This results in hypersensitivity towards drug-induced increases in DA levels, which will facilitate conditioned learning (Volkow *et al.*, 2004; Volkow & Li, 2004), and hyposensitivity towards normal physiological DA increases by natural reinforcers (Volkow *et al.*, 2004) such as food or sex. The Incentive-Sensitization Theory of Addiction is consistent with the hypothesis that chronic drug abuse will cause progressive and perpetual neuroadaptations which will evidently alter behavioural responses (Robinson & Berridge, 1993). Robinson and Berridge (1993) further elaborated on this theory by establishing that chronic drug use will only sensitize the neural systems responsible for inducing the transformation from drug-'wanting' to drug-'craving' and not those responsible for the pleasurable effects of drugs. Drug addiction also accounts for the

neuroplastic alterations and behavioural changes (due to the dysfunction of brain tissues (Leshner, 1997)) observed in addicted patients (DiChiara, 1995). For these reasons drug addiction is now regarded as a neurobiological illness (Leshner, 1997; Yuan *et al.*, 2017).

The characteristic shift of drug-taking behaviour from impulsivity to compulsivity that occurs during addiction involves three different neurobiological circuits and can be divided into three stages (Koob & Franz, 2004) (see figure 2-2):

1. The binge/intoxication stage (illustrated in blue in figure 2-2) during which addictive drugs produce their acute positive and reinforcing effects, mostly by activating the mesocorticolimbic dopaminergic reward system;

2. The withdrawal stage (illustrated in red in figure 2-2) characterized by increased anxiety and negative affect produced by decreases in the function of the extended amygdala. The extended amygdala is responsible for the integration of brain arousal-stress systems with other systems that process sensations that are perceived as pleasurable (hedonic), in order to produce a negative emotional state. However, this stage also involves brain neurochemical systems involved in stress regulation such as the hypothalamic-pituitary-adrenal (HPA) axis and the brain stress system mediated by corticotropin-releasing factor (CRF).

3. The preoccupation/anticipation (illustrated in green in figure 2-2) stage is associated with intense drug craving and involves afferent projections to the nucleus accumbens (NAcc) and amygdala.

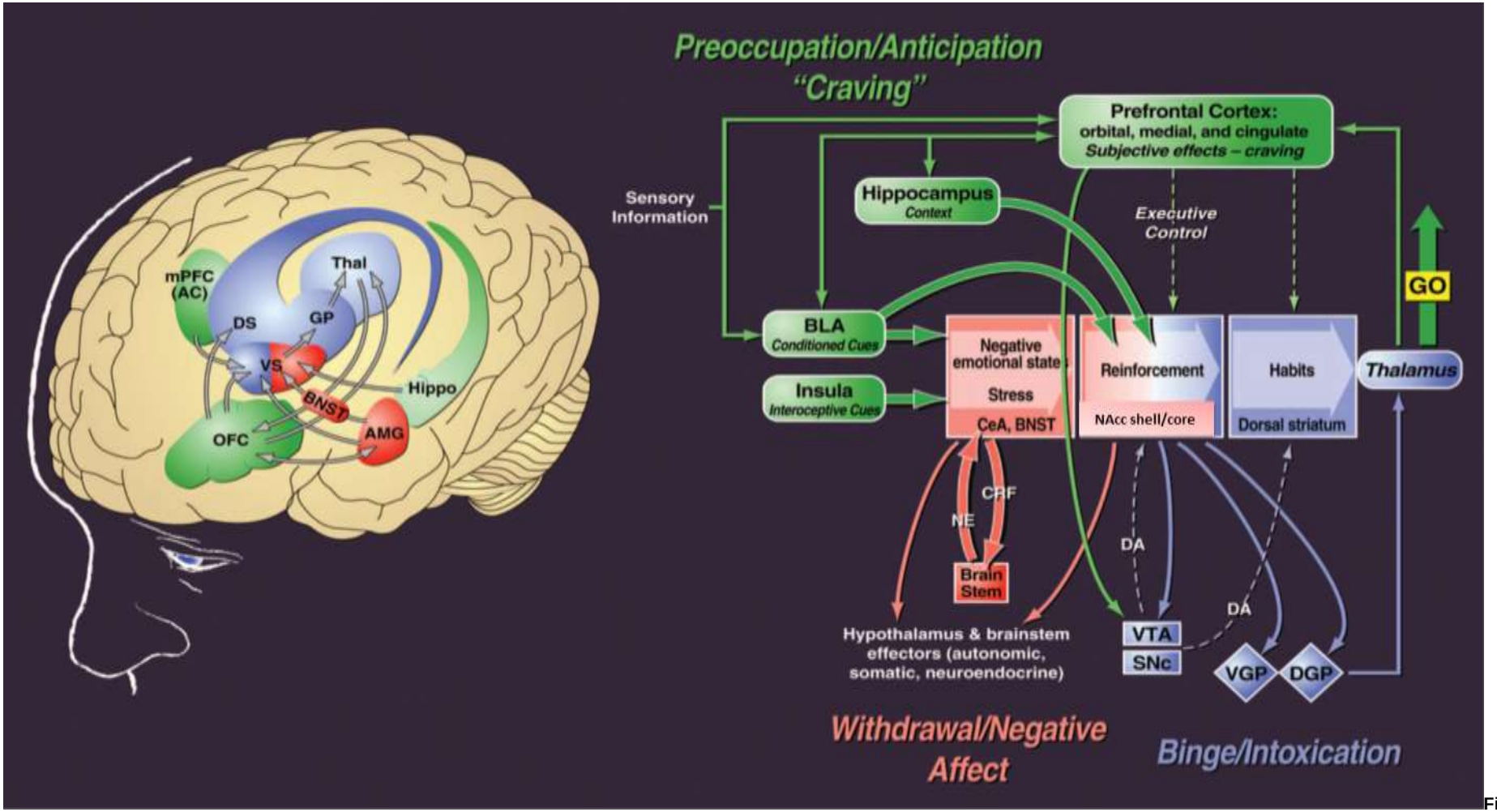


Figure 2-2: Schematic illustration of the neuroadaptations in the brain circuitry during the three stages of the addiction cycle. The ventral striatum/dorsal striatum/extended amygdala is activated by stress from the insula as well as by cues from the hippocampus and basolateral amydala. Deficits in executive function (due to the compromised frontal cortex system) contribute to the perpetual and progressive neuroadaptations caused by chronic drug exposure (incentive salience). Compromised dopamine – and brain stress systems will further contribute to the emergence of an aversive dysphoric state. *AC, anterior cingulate; AMG, amygdala; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CRF, corticotropin-releasing factor; DA, dopamine; DGP, dorsal globus*

pallidus; DS, dorsal striatum; GP, globus pallidus; Hippo, hippocampus; mPFC, medial prefrontal cortex; NAcc, nucleus accumbens; NE, noradrenaline; OFC, orbitofrontal cortex; SNc, substantia nigra pars compacta; Thal, thalamus; VGP, ventral globus pallidus; VS, ventral striatum. Koob and Franz (2004).

2.3.3 Dependence versus addiction

Addiction and dependence are closely related and their definitions are regularly used interchangeably. Therefore, distinction between these two terms will further refine the way in which we study the neurobiology of addiction.

The *Diagnostic and Statistical Manual of Mental Disorders* of the American Psychiatric Association defined drug dependence as "a chronically relapsing disorder that has been characterized by (1) compulsion to seek and take the drug, (2) loss of control in limiting intake and (3) emergence of a negative emotional state" often characterized by irritability, anxiety and dysphoria when drug access is prevented (Koob & Franz, 2004).

Drug dependence is associated with compulsive drug-taking behaviour that represents physical brain activity and is generally characterized by tolerance, physical and psychological dependence (Leonard, 2003; O'Brien & Gardner, 2005).

Tolerance can be defined as an adaptive process in which repeated exposure to a drug warrants the administration of increasing amounts to obtain the desired drug-effect (Leonard, 2003). Repeated exposure to drugs of abuse such as benzodiazepines, opioids and alcohol results in desensitization of the receptors or target sites they act upon in the brain (Leonard, 2003). However, tolerance does not develop for certain stimulant drugs such as cocaine or amphetamines as increasing drug-doses over a prolonged period is not necessary to maintain their euphoric effects (Leonard, 2003).

Physical dependence occurs with continuous exposure to several addictive drugs due to characteristic changes in normal body systems such as increased or decreased receptor densities, enzymatic adaptations and morphological alterations (O'Brien & Gardner, 2005). It is however noteworthy that Leonard (2003) ascribed physical dependence to the abrupt withdrawal of drugs of abuse (such as alcohol, opioids, cannabis, stimulants and cocaine) and Volkow and Li (2004) continued that physical dependence results in withdrawal symptoms produced by body adaptations distinct from those that underlie addiction.

Most drugs of abuse also produce psychological dependence characterized by an initial pleasurable effect with repeated administration and feelings of dysphoria and drug cravings when the drug is suddenly withdrawn (Leonard, 2003).

2.4 Neurocircuitry of drug addiction and reward

It has become clear that drugs of abuse exert their initial effects, whether rewarding or aversive, by activating reward circuits in the brain (Volkow & Morales, 2015), and have the ability to modulate the activity of numerous neurotransmitters, such as DA, opioid peptides, and GABA (Koob & Franz, 2004) with DA being the most affected (Tomkins & Sellers, 2001). Thus, research on the changes in the entire functional system of the neurocircuitry induced by drugs of abuse can provide insight on the complex processes by which they produce their effects. The key reward pathway implicated in the neurocircuitry of drug reward is the mesocorticolimbic dopaminergic pathway (Wise, 2008; Volkow & Morales, 2015) and research on its constituents can provide insightful information on its functioning and rewarding effects.

2.4.1 Mesocorticolimbic dopaminergic system

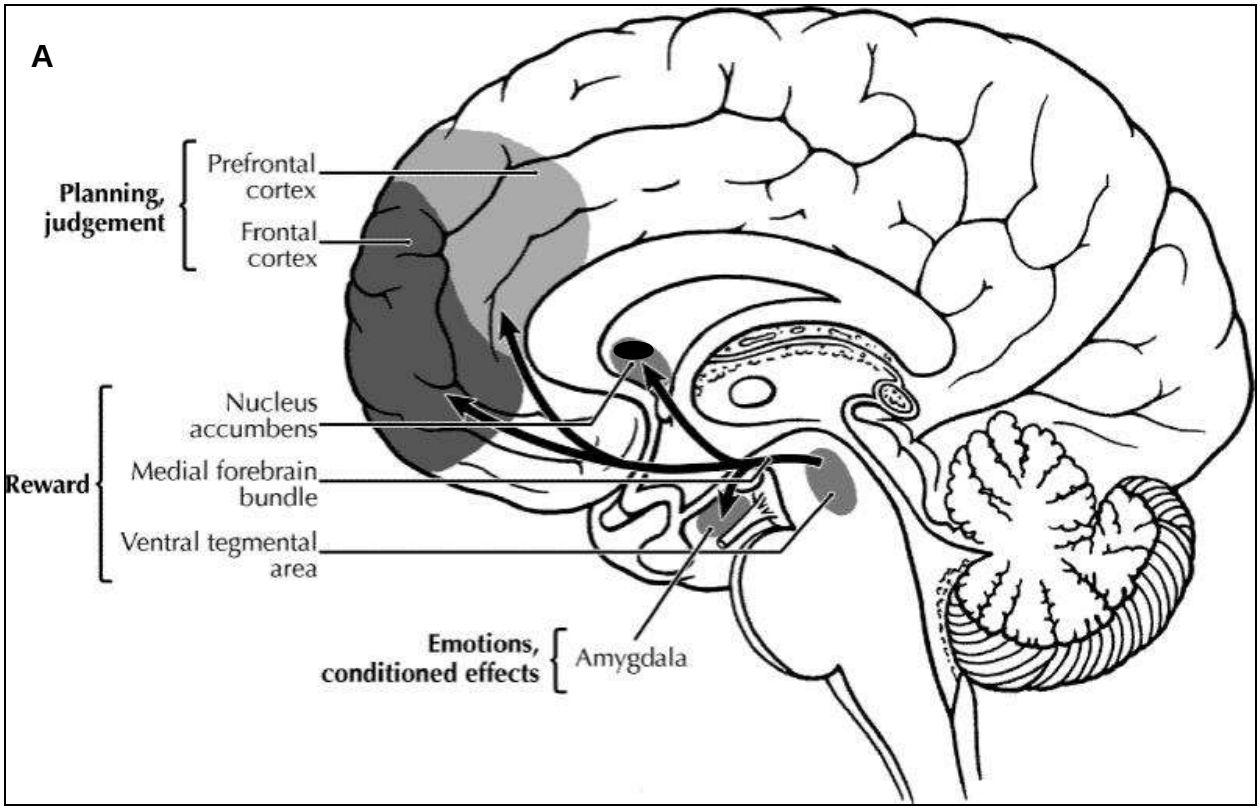
The rewarding effects and consequential addictive profile of most drugs of abuse can be attributed to the disruption of the mesocorticolimbic dopaminergic pathway in the CNS (Poon *et al.*, 2007), which connects the VTA (in the midbrain) to the NAcc (in the ventral striatum) (Ikemoto, 2010) through the medial forebrain bundle (MFB) (see figure 2-3 A) (Tomkins & Sellers, 2001). The MFB contains dopaminergic, serotonergic as well as noradrenergic neurons, however projection of the dopaminergic neurons have been primarily associated with reward (Tomkins & Sellers, 2001). Supporting this finding, several preclinical drug self-administration studies have established that the dopaminergic projection from the VTA to the NAcc plays a particularly important role in reward (Fibiger & Phillips, 1986; Wise & Bozarth, 1987; Koob, 1992; McBride *et al.*, 1999; Pierce & Kumaresan, 2006). Therefore, understanding the interactions between these brain regions and drugs of abuse in inducing their rewarding and consequential addictive properties is essential when evaluating neuronal circuitry in addiction.

The medial section of the mesocorticolimbic dopaminergic pathway can be associated with reward, whilst the lateral section plays a key role in specific conditioned responses (Ikemoto, 2007).

The VTA is an important brain structure localized in the midbrain (Ikemoto, 2010) and can be regarded as a major source of DA in the brain (Ikemoto, 2010). The VTA can be divided into a posterior and anterior region, with the posterior region playing a key role in mediating the rewarding effects associated with the administration of drugs of abuse such as delta-9-tetrahydrocannabinol (Δ^9-THC; principle psychoactive component of *Cannabis sativa*, aka marijuana) (Grinspoon & Bakalar, 1997; Zangen *et al.*, 2006) and the stimulant drug, cocaine (Rodd *et al.*, 2005). Preclinical research on intracranial self-administration supports the finding

that the posterior VTA is more important than the anterior VTA with regards to drug self-administration (Ikemoto, 2010). Moreover, these drugs have the ability to stimulate abundant dopaminergic neurons projecting from the VTA to the striatal complex and predominately to the NAcc (Wise, 2008) (see figure 2-4). These dopaminergic neurons are considered to play a significant role when it comes to the processing of reward induced by drugs of abuse. In correlation with this, optogenetic studies in rats and mice found that the activation of these dopaminergic neurons induces conditioned place preference - a behavioural paradigm used to evaluate the rewarding or aversive effects of drugs and their consequential addictive profiles (Tsai *et al.*, 2009; Witten *et al.*, 2011; Kim *et al.*, 2012). Moreover, these dopaminergic projections in the VTA are surrounded by GABAergic interneurons (Adell & Artigas, 2004) (see figure 2-4). Cocaine administration decreases the firing of these GABAergic interneurons, thereby increasing the excitatory tone in the VTA (see figure 2-4) (Thomas & Malenka, 2003; Steffensen *et al.*, 2008).

The ventral striatum (composed of the NAcc and the striatal part of the olfactory tubercle) is the second and final brain region that forms the mesocorticolimbic dopaminergic pathway (Ikemoto & Bonci, 2014). The NAcc consists of two anatomically heterogeneous elements termed the shell (mainly involved in the incentive motivational properties of rewarding stimuli) and core accumbens (involved in reward-seeking behaviour) (Heimer *et al.*, 1997; Di Chiara, 2002; Russo *et al.*, 2010) (see figure 2-3 B).



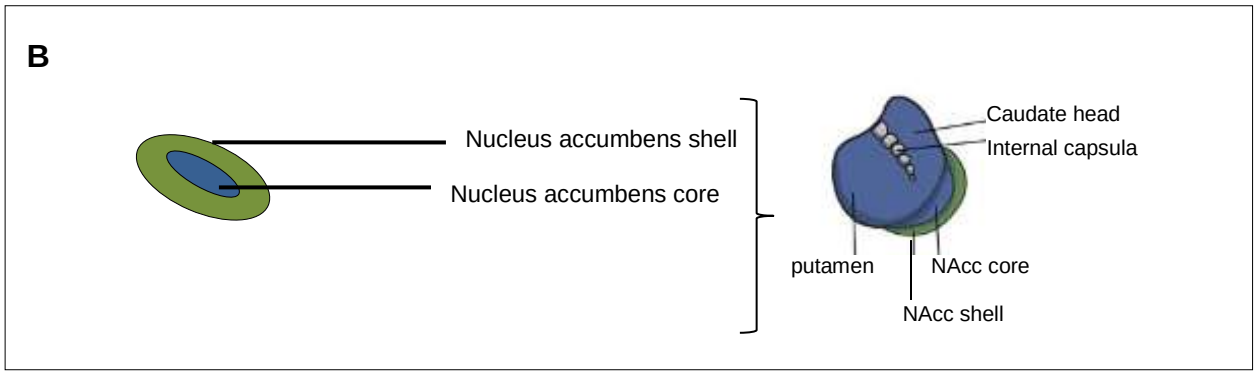


Figure 2-3 (A): Schematic illustration of the mesocorticolimbic dopaminergic pathway connecting VTA to the NAcc through the MFB which is implicated in reward processes together with the prefrontal and frontal cortex implicated in executive functions such as planning and judgement and the amygdala implicated in specific conditioned responses. Adapted from Tomkins and Sellers (2001). (**B)**: Schematic illustration of the NAcc shell and core implicated to play a significant role in incentive motivational properties and reward-seeking behaviour respectively.

The NAcc mainly consists of GABAergic projection neurons and interneurons which project to, amongst others, the VTA (Koob & Volkow, 2010; Rutherford *et al.*, 2011). As previously mentioned, the NAcc receives dopaminergic afferents projecting from the VTA (see figure 2-4)as well as glutamatergic afferents projecting from the midline thalamus and limbic cortices (Heimer, 1975; Heimer, 1978). Owesson-White *et al.* (2009) reported that drugs of abuse imitate the phasic firing of dopaminergic neurons, thereby increasing DA release in the shell accumbens. This supraphysiological surge in DA release will then activate the direct striatal pathway via dopamine D_1 receptors and inhibit the indirect striatal pathway via dopamine D_2 receptors (Volkow & Morales, 2015). D_1 receptor activation on the direct striatal pathway is associated with reward and can induce associative learning (Zweifel *et al.*, 2009), whilst inhibition of the indirect pathway via D_2 receptors is associated with a decrease in the aversive responses towards drug of abuse (Hikida *et al.*, 2010; Kravitz *et al.*, 2012). Taking this into account, Volkow and Morales (2015) concluded that optimal drug reward and drug-conditioning can be observed when both D_1 and D_2 receptors are activated.

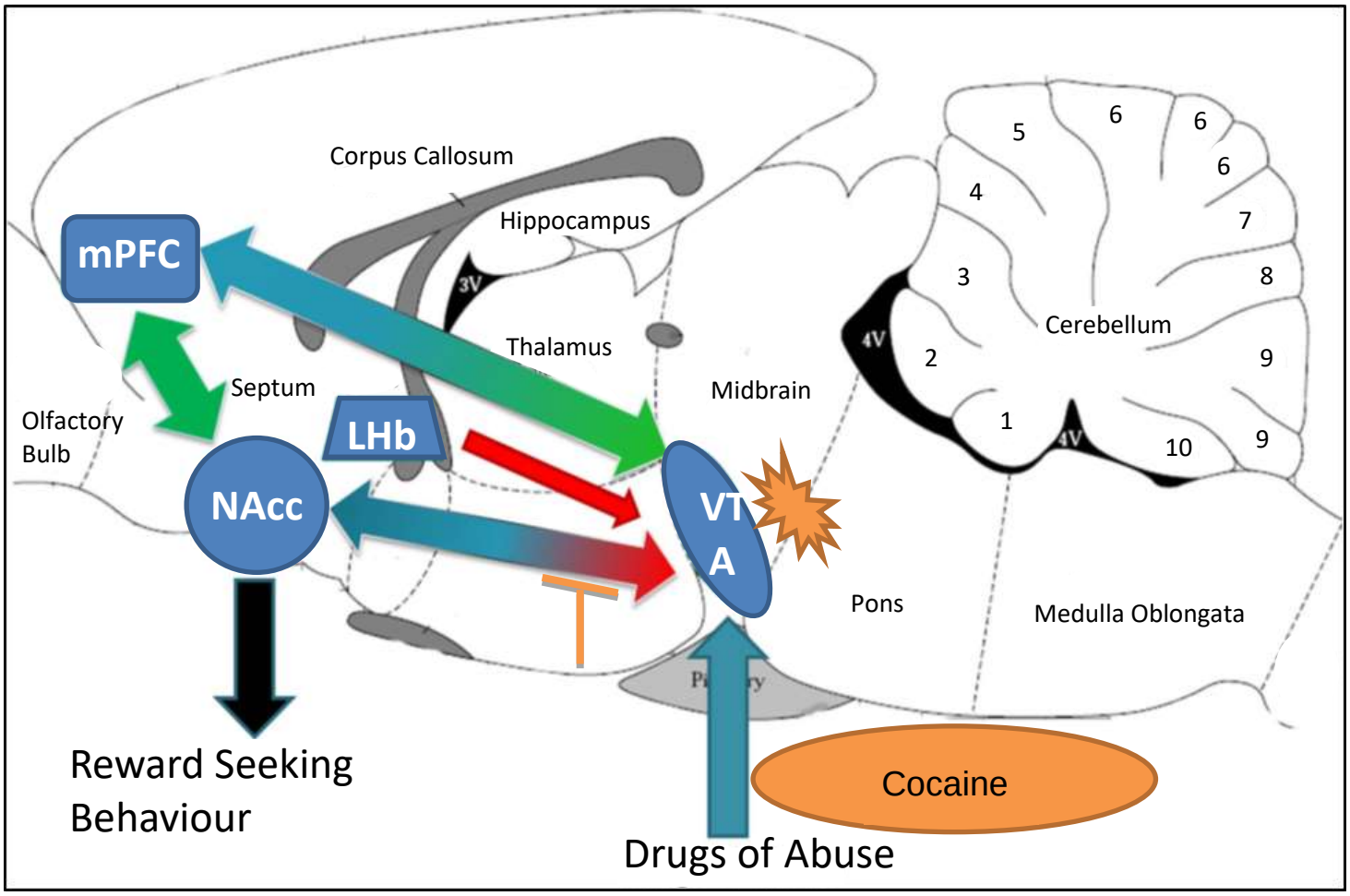


Figure 2-4: Mesocorticolimbic pathway also illustrating projections to the medial prefrontal cortex (mPFC) in the rat brain. The ventral tegmental area (VTA) sends dopaminergic projections (blue arrows) to the nucleus accumbens (NAcc) and medial prefrontal cortex (mPFC). The VTA is also innervated with GABAergic projections from the NAcc (as well as the lateral habenula (LHb)) (red arrows) and glutamatergic projections from the mPFC (green arrows). Notably, the administration of cociane will inhibit the firing of these GABAergic neurons, which will result in an excitatory state in the VTA (indicated in orange). The mPFC and NAcc have reciprocal glutamatergic projections and any alteration in the activity of the NAcc will lead to reward seeking behaviour (black arrow). Adapted from Rutherford *et al.* (2011).

The enduring alterations in the mesocorticolimbic dopaminergic system produced by drugs of abuse can, in turn, alter certain behaviours, including those of a social nature (Young *et al.*, 2011). In particular, the hypothalamic hormone, oxytocin (OT) and its interactions with several neurocircuitry pathways, especially the mesocorticolimbic dopaminergic pathway, are involved in various social and anxiety-like behaviours (Zimmer, 2015). Deeper investigation into OT and

the effects of drugs of abuse on this hypothalamic hormone can provide significant insight into certain behaviours provoked by drugs of abuse.

2.4.2 Oxytocin

OT is a nine-amino acid peptide hormone and neuromodulator synthesized in the neurosecretory cells of the paraventricular nucleus (PVN), supraoptic nucleus (SON) as well as the accessory nuclei (AN) of the hypothalamus (Sofroniew, 1983; Swanson & Sawchenko, 1983; McGregor *et al.*, 2008). OT is released into the circulatory system via the posterior pituitary to produce its peripheral effects (such as uterine contractions during labour and the milk ejection reflex) (Janeček & Dabrowska, 2018), or it diffuses locally within the hypothalamus (Landgraf & Neumann, 2004; Ludwig & Leng, 2006; Neumann, 2007) to produce its central effects. Some of OT's most relevant central effects include the promotion of peer-to-peer social interaction and social memory as well as the reduction in anxiety responses (McGregor *et al.*, 2008). For these reasons the central release of OT has been implicated in 'pro-social' and anxiolytic behaviour (McRae-Clark *et al.*, 2013). Preclinical research in rats also demonstrated that OT neurons from the PVN send projections to limbic brain regions (such as the VTA), the main olfactory bulb (MOB), amygdala, medial preoptic area of the hypothalamus (MPOA) and hypothalamus, all brain structures involved in the modulation of anxiety-like behaviours (McGregor *et al.*, 2008; Dabrowska *et al.*, 2011; Rutherford *et al.*, 2011; Knobloch *et al.*, 2012) (see Figure 2-4). The VTA receives oxytonergic projections from the PVN which then innervate dopaminergic neurons that project to the NAcc to produce profound social effects and increases in mesolimbic DA activity (Melis *et al.*, 2007; McGregor *et al.*, 2008). Clinical studies further investigated the reported 'pro-social' and anxiolytic effects of OT and found that intranasally administered OT improved the recognition of social cues, increased trust and gaze in the direction of the eye regions of other individuals and it decreases amygdala activation towards fear-inducing stimuli (Kirsch *et al.*, 2005; Kosfeld *et al.*, 2005; Domes *et al.*, 2007; Guastella *et al.*, 2008; McGregor *et al.*, 2008).

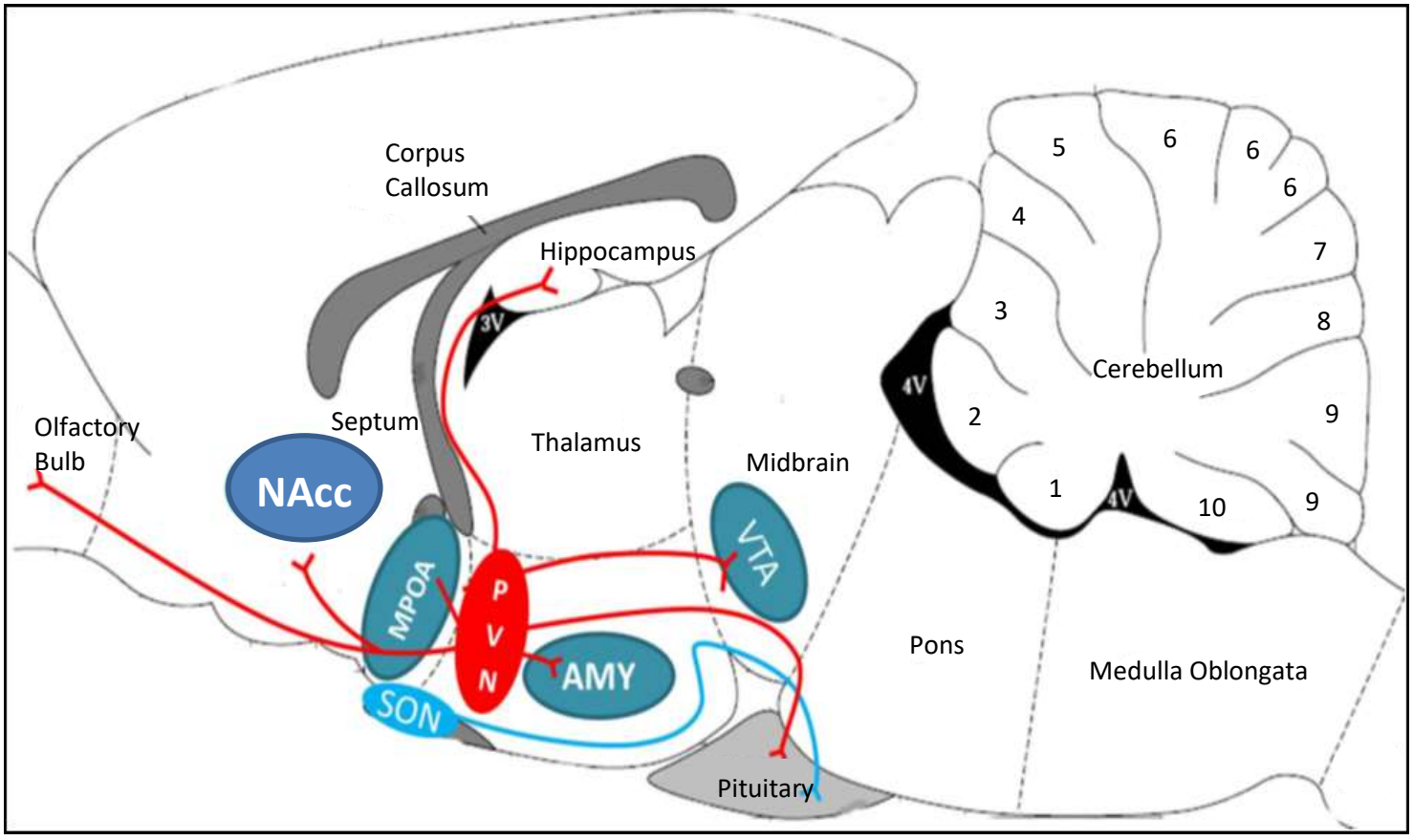


Figure 2-5: Oxytonergic projection in the rat brain. The peripheral release of oxytocin is mediated by magnocellular neurons from the supraoptic nucleus (SON) (blue oval) and PVN (red oval) projecting to the posterior pituitary. The parvocellular neurons of the PVN also project to several neurocircuits viz. the VTA and NAcc (rewarding circuits), the stress circuits (composed of the hippocampus and AMY) and maternal circuits (MPOA and olfactory bulb). *SON, supraoptic nucleus of the hypothalamus; PVN, paraventricular nucleus of the hypothalamus; VTA, ventral tegmental area; Nacc, nucleus accumbens; AMY, amygdala; MPOA, medial preoptic area of the hypothalamus* (Rutherford *et al.*, 2011).

It is of particular importance that OT is a key modulator of both natural and drug-induced reward pathways (Leong *et al.*, 2016). Zhou *et al.* (2008) reported that OT may play an essential role in social and other behavioural processes relating to drug addiction. For example, low doses 3,4-methylenedioxymethamphetamine (MDMA) increased the initial social interaction between unfamiliar rat pairs (Morley & McGregor, 2000; Thompson *et al.*, 2007). This effect is most likely produced by MDMA-induced OT release in the hypothalamus, considering that low doses MDMA activate OT-containing neurons in the SON and PVN of rats, thereby increasing their plasma OT levels (Thompson *et al.*, 2007). This interaction reflects the merging action of 5-HT-containing terminals and OT-containing cell bodies within the PVN and SON (McGregor *et al.*, 2008). MDMA exposure not only alters behaviour of a social nature, preclinical studies in rats also showed that pre-exposure to MDMA produces prolonged increases in anxiety, impaired

memory and coping responses to acute stress (Morley *et al.*, 2001; McGregor *et al.*, 2003; Thompson *et al.*, 2004). Yoshida *et al.* (2009) ascribed the 'pro-social' and anxiolytic properties of OT to enhanced 5-HT neurotransmission. Very recent *in vivo* studies by Chruścicka *et al.* (2018) showed a potential link between 5-HT_{2A} – and OT receptors and their neurotransmitters (5-HT and OT) taking into account that an anxiolytic effect is produced by OT receptors expressed in serotonergic neurons, while OT administration causes changes in 5-HT innervation. The exact mechanism on how these receptors and their neurotransmitters affect each other is unknown and warrants further research. In correlation with the above, Sarnyai (2011) suggested that OT (in relationship with 5-HT and DA) may possibly promote the 'pro-social' effects produced by illicit drugs as seen in individuals addicted to various psychostimulants. Acute cocaine exposure causes a dose-dependent increase in OT release in the hypothalamus and hippocampus (Kovács *et al.*, 1998), whilst chronic cocaine exposure significantly decreased OT levels in the hippocampus, VTA and MPOA of female rats in preclinical studies evaluating the effects of cocaine exposure on maternal behaviour (Johns *et al.*, 1997). Decreased OT levels were also detected in the plasma, hippocampus and hypothalamus of male rats exposed to chronic cocaine administration (Sarnyai *et al.*, 1992), suggesting that gender differences do not affect the role of OT in different addiction potentials towards at least cocaine as a drug of abuse. The anxiolytic properties of gamma-hydroxybutyrate (GHB) (also defined as a 'pro-social' drug) (Schmidt-Mutter *et al.*, 1998) can also be attributed to its ability to activate OT containing neurons in the SON (McGregor *et al.*, 2008). On the other hand, chronic exposure to low dose Δ^9-THC decreases OT receptor densities and OT innervation in the NAcc of rats (Butovsky *et al.*, 2006), which can possibly explain the reported prolonged social interaction deficits seen in rodents exposed to cannabinoids (O'Shea *et al.* (2004); O'Shea *et al.* (2006); Quinn *et al.* (2008)).

OT also interacts with the endogenous cannabinoid system, another neurocircuit involved in reward as well as stress and anxiety responses (Viveros *et al.*, 2005) in response to drugs of abuse.

2.4.3 The Endogenous Cannabinoid (Endocannabinoid) System

The endocannabinoid signalling system primarily consists of cannabinoid (CB) receptors and endogenous cannabinoids [such as anandamide and 2-arachidonoylglycerol (Devane *et al.*, 1992; Di Marzo *et al.*, 1994; Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995; Stella *et al.*, 1997)]. The latter are only synthesized and released on demand (van der Stelt & Di Marzo, 2003) and act as retrograde messengers to inhibit the release of several neurotransmitters (Ralevic, 2003). Both endogenous and exogenous cannabinoids (such as Δ^9-THC) produce their characteristic physiopsychological effects by interacting with CB receptors, which are present in relatively

large quantities in the hippocampus, cerebellum and prefrontal cortex (PFC) where they act toy influence memory, motor co-ordination and affective states respectively (Herkenham *et al.*, 1990; Tsou *et al.*, 1998). Two types of CB receptors have been identified: CB_1 (Herkenham *et al.*, 1990; Herkenham *et al.*, 1991) and CB_2 receptors (Munro *et al.*, 1993). CB_2 receptors are primarily expressed on immune cells in the peripheral system and at low levels on some neurons within the brain (Van Sickle *et al.*, 2005; Wotherspoon *et al.*, 2005; Beltramo *et al.*, 2006; Gong *et al.*, 2006). Due to the widely reported scarcity of CB_2 receptors in the central nervous system (Atwood & Mackie, 2010), we will mainly focus on the neuronal role of CB_1 receptors, The CB_1 receptor subtype is abundantly expressed in the brain (Herkenham *et al.*, 1990) and is predominantly situated at the axon terminals of both central and peripheral neurons (Pertwee, 2008) and accounts for most of the effects induced by cannabis. CB_1 receptors are present in higher densities in the striatum than in the VTA (Herkenham *et al.*, 1990; Tsou *et al.*, 1998). However, despite the low CB_1 concentrations in the VTA, cannabinoids still produce rewarding effects when injected directly into this region (Szabo *et al.*, 2002; Cheer *et al.*, 2003; Melis *et al.*, 2004; Riegel & Lupica, 2004).

CB_1 receptors are metabotropic G-protein coupled receptors (Howlett *et al.*, 2002). Stimulation of presynaptic CB_1 receptors will inhibit the release of neurotransmitters such as DA, 5-HT, NE, acetylcholine, GABA and glutamate (Howlett *et al.*, 2002; Pertwee & Ross, 2002; Szabo & Schlicker, 2005) through inhibition of the adenylate cyclase-cyclic adenosine monophosphate cascade, closure of calcium channels and opening of potassium channels as well as stimulation of kinases (Howlett *et al.*, 1998; McAllister & Glass, 2002).

A preclinical study conducted by Tanda *et al.* (1997) established that cannabinoids increase DA-release in the shell region of the NAcc. Furthermore, the stimulation of CB_1 receptors situated on GABAergic terminals in the VTA will also increase DA release in the NAcc by stimulating the firing rates of the DA neurons in the VTA which project into the NAcc (French, 1997; French *et al.*, 1997; Gessa *et al.*, 1998; Cheer *et al.*, 2000; Wu & French, 2000; Cheer *et al.*, 2003). What is of particular significance is that Cheer *et al.* (2000) found that this effect was blocked by the administration of a $GABA_A$ receptor antagonist, indicating that cannabinoids decrease the inhibitory effect of GABA on dopaminergic neurons and thereby increasing DA release (Pierce & Kumaresan, 2006). Cannabinoids also inhibit excitatory glutamate transmission through a presynaptic mechanism which could possibly counteract the above-mentioned effect of cannabinoids on inhibitory GABAergic tone situated on dopaminergic neurons (Melis *et al.*, 2004).

2.5 Neurochemistry and consequential behavioural alterations induced by drugs of abuse

In order to evaluate EFV's possible abuse potential, one should review and compare the neurochemistry of EFV together with known drugs of abuse. Comparable drugs with regards to EFV's abuse potential include:

- LSD: A recent study found that EFV's effects are very similar to that of LSD and is primarily mediated via its weak, partial agonistic action on the 5-HT_{2A} receptor subtype (Gatch *et al.*, 2013; Dalwadi *et al.*, 2016).
- Δ^9-THC: Δ^9-THC is relevant in the evaluation of EFV's abuse potential since it is one of the key components in the potent "Nyaope" drug-cocktail (Rough *et al.*, 2014).
- MA: As previously mentioned, MA is reported to be present in the potent "Nyaope"-cocktail (Rough *et al.*, 2014), and could possibly account for some of the neuropsychiatric effects seen in "Nyaope" addicts. A review by Halpin *et al.* (2014) stated that MA has been noted to provoke oxidative stress that appears to underlie both it psychogenic and neurotoxic properties. EFV too has been found to be a prooxidant also purported to mediate its psychotropic actions (Dalwadi *et al.*, 2018).

2.5.1 Lysergic acid diethylamide (LSD)

LSD belongs to the indoleamine class of serotonergic hallucinogens (Halberstadt & Geyer, 2011) and is a well-known drug of abuse with strong psychomimetic effects, viz. hallucinations (Ouagazzal *et al.*, 2001b; Marona-Lewicka *et al.*, 2011; Martin *et al.*, 2014) and delusions comparable to the positive symptoms of schizophrenia (Breier, 1995). LSD intoxication induces profound alterations in affective states and responses, including that related to mood, intuition, thought, sensory perception and the relative experience of time and space (Passie *et al.*, 2008), by producing significant distortions of these perceptual processes (Halberstadt & Geyer, 2011). These ubiquitous effects of LSD implicate the involvement of the cerebral cortex (Aghajanian & Marek, 1999a).

Freedman (1984) reported two distinct temporal phases 4-6 hours after LSD-administration in humans. The first phase consists of a "psychedelic experience" which can be attributed to LSD's high affinity interactions with 5-HT receptors in the brain (Minuzzi *et al.*, 2005). The distinctive hallucinogenic properties and reported stimulus effect (Glennon, 1986) of LSD are substantially mediated by the activation of the serotonin 5-HT_{2A} receptor subtype, (Nichols, 2004; Halberstadt & Nichols, 2010). Several studies have found that 5-HT_{2A} receptor activation will lead to an increase in glutamate levels in the cortex (Martín-Ruiz *et al.*, 2001; Winter *et al.*,

2004) This is of particular relevance since increased extracellular glutamate levels in the PFC have been found to induce psychosis-like symptoms (Marona-Lewicka *et al.*, 2011). Thus, the release of glutamate can be seen as the final result of the actions of both serotonergic and glutamatergic hallucinogens (Aghajanian & Marek, 1999b). Preclinical research has reported that LSD disrupts pre-pulse inhibition (PPI) of the acoustic startle reflex in rats (Ouagazzal *et al.*, 2001a). PPI is used to measure sensorimotor gating, a neurological filtering process by which insignificant or excessive stimuli are either screened or 'filtered' out of awareness to prevent information overload to the brain (Braff & Geyer, 1990; Arai *et al.*, 2008). Sensorimotor gating is an important clinical and preclinical behavioural marker that provides insight into the monoaminergic changes and attentional abnormalities observed in psychotic patients (Braff & Geyer, 1990) and may also provide insight on the developmental mechanism of psychosis (Cadenhead, 2011). Importantly, LSD-induced disruption in PPI was blocked by the administration of 5-HT_{2A} antagonists (Sipes & Geyer, 1995; Ouagazzal *et al.*, 2001a; Halberstadt & Geyer, 2010), reaffirming the important role for 5-HT_{2A} receptors in decreasing PPI. These data therefore support the hypothesis that LSD (and other 5-HT_{2A} agonists) produce similar psychotic states as that seen in schizophrenia-patients (Passie *et al.*, 2008). Another study reported that LSD administration in rats produces a biphasic effect on locomotor behaviour where locomotor activity is initially suppressed, but increases over time due to habituation to the novel environment where locomotor activity is assessed (Mittman & Geyer, 1991).

Several preclinical studies also found that LSD inhibits the firing of serotonergic neurons in the dorsal raphe nuclei by stimulating the serotonin 5-HT_{1A} auto receptors. Liebsch *et al.* (1998) reported that the stimulation of 5-HT_{1A} receptors correlates with increased anxiety in rats. LSD also interacts with the serotonin 5-HT_{2C} receptor subtypes (Yagaloff & Hartig, 1985; Peroutka, 1994; Egan *et al.*, 1998) that have been associated with anxiogenic responses (Kennett *et al.*, 1997).

Although the serotonergic mechanism of action of LSD is predominately responsible for its hallucinogenic profile and can explain important neurochemical interactions, there is also extensive evidence supporting the possibility that LSD induces its psychopharmacological and behavioural effects through its interaction with other non-serotonin receptors such as dopaminergic and adrenergic receptors (Watts *et al.*, 1995; Minuzzi *et al.*, 2005; Halberstadt & Geyer, 2011). In this regard, the interaction between LSD and DA receptors produces significant changes in the synthesis, release and metabolism of DA (Da Prada *et al.*, 1975; Persson, 1977; Persson, 1978; Pieri *et al.*, 1978) as well as DA cell firing (Christoph *et al.*, 1977; Walters *et al.*, 1979). Halberstadt and Geyer (2011) reported that LSD's dopaminergic

action is essential in mediating the behavioural effects of the drug in the drug discrimination paradigm. This is of relevance considering that the second temporal phase (characterized by a clear paranoid state) (Freedman, 1984) is mediated by the activation of central dopamine D_2 receptors (Marona-Lewicka & Nichols, 2007) where LSD acts as a partial albeit high affinity agonist (Walters *et al.*, 1979; Watts *et al.*, 1995; Nichols *et al.*, 2002). LSD also binds to D_1 receptors where it acts as a partial agonist and which also may underlie its psychoactive effects (Watts *et al.*, 1995).

2.5.2 Δ^9-Tetrahydrocannabinol (Δ^9-THC)

Δ^9-THC acts as a partial agonist at cannabinoid CB_1 and CB_2 receptors. However, a previous study found that Δ^9-THC can both activate or antagonize CB receptors depending on the density and coupling efficiencies of these receptors (Pertwee, 2008) and whether Δ^9-THC acts as an agonist or antagonist at these receptors will determine the behavioural response that it elicits.

2.5.2.1 Varied mechanism of action of Δ^9-THC and its effects on anxiety-like behaviour

The effects of cannabinoids, and Δ^9-THC in particular, on anxiety-responses are complex and seems to be dependent on several factors including the density and coupling efficiencies of the CB_1 receptors, the stimulatory-inhibitory effect of Δ^9-THC on central neurotransmitters (Pertwee, 2008) as well as the Δ^9-THC-dose (Berrendero & Maldonado, 2002). The CB_1 receptor density and coupling efficiencies vary significantly between different brain areas and species. For example, human brains express more CB_1 receptors in the amygdala and less in the cerebellum when compared to a rat's brain (Herkenham *et al.*, 1990; Pertwee, 2008). This could explain the enhanced effects of CB_1 agonists on motor function observed in rats when compared to humans (Herkenham *et al.*, 1990). Haller *et al.* (2007) also supported the species difference theory by proving that the administration of the CB_1 receptor agonist, R-(+)-WIN55212, caused anxiogenic responses in rats but induced anxiolytic behaviour in mice. Furthermore, in the presence of low-expression levels or low coupling efficiencies of CB_1 receptors, Δ^9-THC has the ability to act as an antagonist rather than an agonist on CB_1 receptors whilst the opposite is true for brain areas with high CB_1 receptor densities and coupling efficiencies (Patel & Hillard, 2006; Pertwee, 2008). When Δ^9-THC acts as an antagonist it has the ability to produce anxiogenic effects similar to those induced by the CB_1-selective antagonists SR141716A and *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251), whilst the administration of the CB_1 agonist R-(+)-WIN55212 induced anxiolytic behaviour (Patel & Hillard, 2006). Interestingly, one preclinical study also proposed that the effects of Δ^9-THC on anxiety responses are dose-dependent seeing that rats exposed to low dosages Δ^9-THC exhibited anxiolytic behaviour, whereas higher dosages produced anxiogenic activity (Berrendero & Maldonado, 2002).

2.5.2.2 The varied stimulatory-inhibitory effect of Δ^9-THC on neurotransmitter-release

CB_1 receptors mediate the inhibition of the release of several neurotransmitters (such as DA, 5-HT, NA, acetylcholine, GABA and glutamate) from the neurons on which they are expressed (Howlett *et al.*, 2002; Pertwee & Ross, 2002; Szabo & Schlicker, 2005; Pertwee, 2008). However, several preclinical studies have reported that in vivo administration of Δ^9-THC (and Δ^9-THC-mediated activation of the G-protein coupled CB_1 receptor) causes an increase in the release of these neurotransmitters with increases in acetylcholine, DA and glutamate release in the prefrontal cortex of rats, increases in the release of acetylcholine in the rat hippocampus and increases in DA release in the NAcc of rats (Pertwee & Ross, 2002; Pistis *et al.*, 2002; Gardner, 2005; Nagai *et al.*, 2006; Pisanu *et al.*, 2006). These increases in DA, glutamate or acetylcholine can, in part, be attributed to the direct or indirect inhibition by Δ^9-THC of inhibitory DA-, acetylcholine- and glutamate-release through activation of CB_1 receptors (Pertwee, 2008).

To better explain, Δ^9-THC stimulates CB_1 receptors to inhibit glutamate-release onto inhibitory GABAergic neurons, which project from the NAcc to the VTA where they inhibit the release of DA from neurons projecting back to the NAcc (Pertwee & Ross, 2002) (see figure 2-6). Thus, the inhibitory effect of CB_1 receptor activation attenuates DA-release in the NAcc (Pertwee & Ross, 2002) (see figure 2-6) and decreases the interneuronal release of GABA (Katona *et al.*, 1999; Sullivan, 1999) resulting in disrupted synchronization of pyramidal cell activity (Hoffman & Lupica, 2000; Wilson & Nicoll, 2002), which is hypothesized to play a significant role in cognition and sensory perceptions (Wilson & Nicoll, 2002). These cognitive and sensory disruptions could induce deficits in sensorimotor gating mechanisms (Todd *et al.*, 2017) and eventually lead to psychotic symptoms (D'Souza *et al.*, 2004).

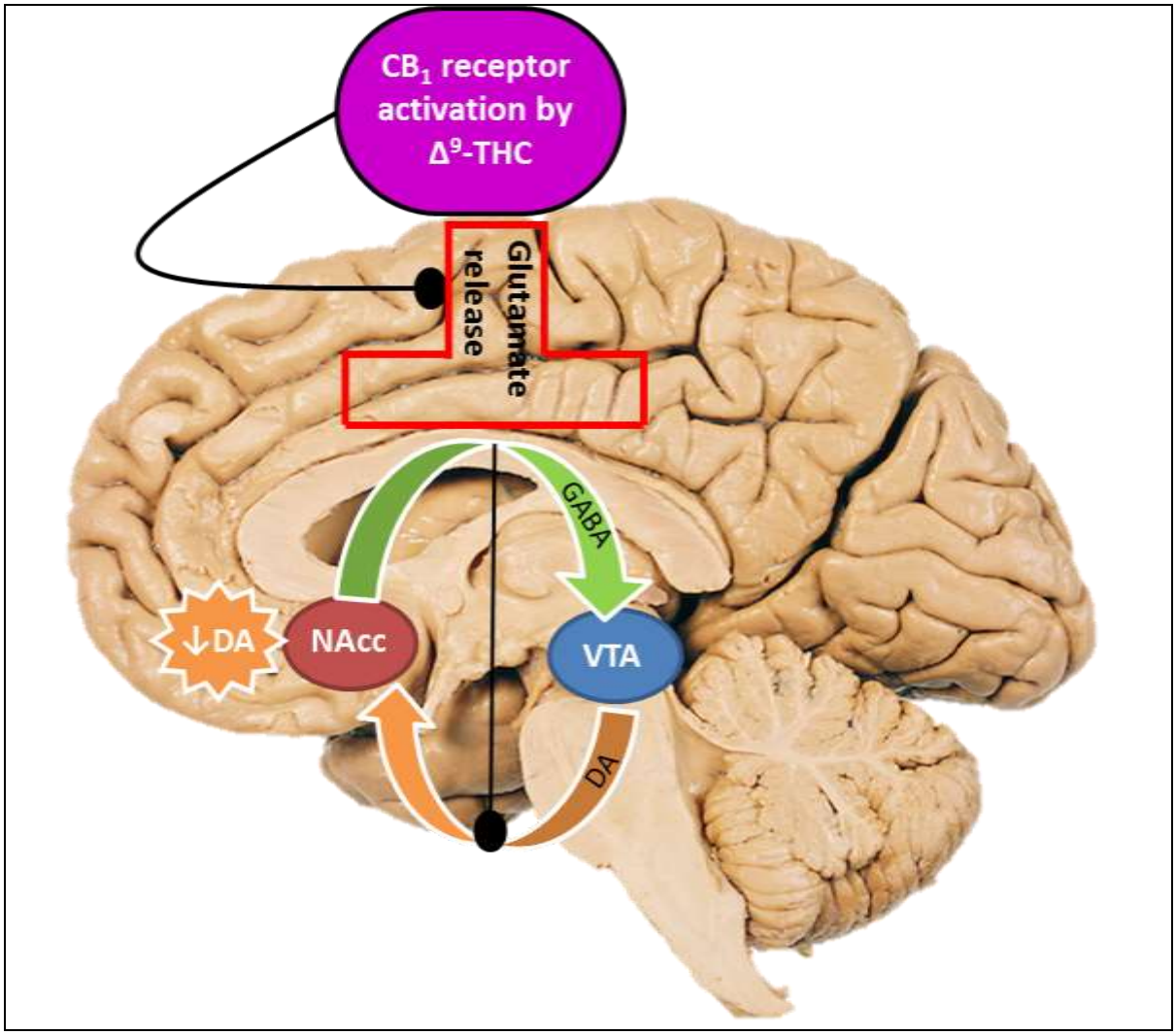


Figure 2-6: The effect of Δ^9-THC-mediated CB_1 receptor activation on central neurotransmitters. Δ^9-THC stimulates CB_1 receptors to inhibit the release of excitatory glutamate (shape with red outline) onto inhibitory GABAergic neurons (green arrow) that project from the VTA to the NAcc where they inhibit the firing of dopaminergic neurons (orange arrow) that project back to the NAcc. This will lead to a significant decrease in DA release in the NAcc. *VTA, ventral tegmental area; NAcc, nucleus accumbens.*

Other preclinical studies revealed that Δ^9-THC-mediated CB_1 receptor activation causes a significant elevation in striatal DA levels (Malone & Taylor, 1999). Malone and Taylor (1999) suggested the involvement of serotonergic mechanisms in mediating striatal DA release, considering that the selective serotonin reuptake inhibitor, fluoxetine, abolished the Δ^9-THC-mediated DA release (Malone & Taylor, 1999). Several other studies also reported that CB_1 receptor activation by Δ^9-THC enhanced neuronal firing of mesolimbic DA neurons (French, 1997; French *et al.*, 1997; Tanda *et al.*, 1997; Gessa *et al.*, 1998; Malone & Taylor, 1999). D'Souza *et al.* (2004) reported that increased mesolimbic dopaminergic activity will result in symptoms such as paranoia, illusions, depersonalization, derealisation and even distorted sensory perceptions. Stimulation of CB_1 receptors also activates DA transmission in the PFC resulting in deficits in working memory and other cognitive processes (D'Souza *et al.*, 2004).

These increased DA levels could account for the rewarding effect induced by Δ^9-THC. For example, rats receiving Δ^9-THC showed a clear preference for the Δ^9-THC-paired chamber of the conditioned place preference apparatus, indicative of its possible addictive and rewarding effects (Braida *et al.*, 2004; Justinova *et al.*, 2005).

Δ^9-THC-mediated CB_1 receptor activation also increases acetylcholine release in the PFC through a 'disinhibitory' process that involves the inhibition of GABA release onto acetylcholine-releasing neurons (Pertwee & Ross, 2002).

Furthermore, the activation of CB_1 receptors by Δ^9-THC will also reduce the synaptic transmission of glutamate through a presynaptic mechanism in the hippocampus and cerebellum (Shen *et al.*, 1996; Levenes *et al.*, 1998), that could affect behavioural responses related to anxiety (Rey *et al.*, 2012).

In conclusion, the mixed mechanism of action of Δ^9-THC as well as its effect on several neurotransmitters in the CNS can possibly explain the controversy that exists on some of the behavioural responses (with particular regards to anxiety), taking into account that several preclinical studies found that Δ^9-THC has the ability to induce anxiolytic behaviour in some studies but increase anxiety in others (Berrendero & Maldonado, 2002; Patel & Hillard, 2006; Braida *et al.*, 2007; Schramm-Sapyta *et al.*, 2007).

2.5.3 Methamphetamine (MA)

MA, a synthetic derivative of amphetamine, is commonly abused for its sympathomimetic effects on the CNS resulting in enhanced mental alertness, euphoria and increased physical activity (Tata & Yamamoto, 2007), with all these factors contributing significantly to its abuse-potential. MA also exerts profound neurotoxic effects when abused over prolonged periods (Volkow *et al.*, 2001a; Volkow *et al.*, 2001b; Darke *et al.*, 2008). Internationally, MA-abuse is increasing at an alarming rate (Nordahl *et al.*, 2003), emphasizing the need for understanding the precise mechanism(s) by which it exerts its neurotoxic effects. It is postulated that MA induces alterations in behavioural and cognitive processes by altering monoaminergic systems (Nordahl *et al.*, 2003). MA-induced neurological damage (such as dysfunction of the frontal lobe) leads to impaired social-cognitive functioning (Homer *et al.*, 2008). Paranoia, social withdrawal and anxiety have been well documented in human MA abusers (Davidson *et al.*, 2001; Rawson *et al.*, 2002), whilst Clemens *et al.* (2004) reported significant social anxiety in rats receiving MA.

It has been reported that acute MA-administration is associated with increased extracellular DA concentrations (Nordahl *et al.*, 2003). MA-exposure promotes DA-release either by inhibiting

GABA receptors in the VTA through the activation of μ-opoid receptors (Stephans & Yamamoto, 1994), through reverse transport of DA or by the displacement of DA from DA-stores (Liang & Rutledge, 1982; Sulzer *et al.*, 1993). Previous preclinical studies observed that chronic MA exposure reduces DA concentrations in the striatum (Wagner *et al.*, 1980; Ricaurte *et al.*, 1984; Seiden, 1987). However, a previous preclinical study conducted in our laboratory reported unaltered striatal DA levels, but increased frontal-cortical DA levels, following an escalating chronic dose regime of MA exposure (Swanepoel *et al.*, 2018). A preclinical study conducted by Abekawa *et al.* (1996) reported decreased levels of DA and its metabolite - dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striatum following the administration of toxic MA doses. This phenomenon could be explained by the formation of nitric oxide (NO) which is known to modulate DA release (Hanbauer *et al.*, 1992; Bugnon *et al.*, 1994). In fact the co-administration of Nω-nitro-L-arginine methyl ester (which inhibits the nitric oxide synthase enzyme - the enzyme responsible for the production of NO from L-arginine (Andrew & Mayer, 1999)) was found to lower the striatal DA, DOPAC and HVA reductions induced by MA (Abekawa *et al.*, 1996).

DA transporters (DAT) may also play a significant role in the ability of MA to induce its reported neurotoxic effects (Fumagalli *et al.*, 1998), which is characterized by marked and long-term depletions in striatal DA and 5-HT (Tata & Yamamoto, 2007). Preclinical studies reported that the neurotoxic effects of MA lead to neurodegeneration, which is evident in MA-exposed subjects who had reduced DAT activity (Fumagalli *et al.*, 1998), damaged DA nerve terminals (Ricaurte *et al.*, 1982), reduced activity of DA's rate-limiting biosynthetic enzyme - tyrosine hydroxylase - as well as decreased vesicular monoamine transporter-2 (VMAT2) protein levels (Hotchkiss & Gibb, 1980; Schmidt & Gibb, 1985; Nordahl *et al.*, 2003). VMAT2 is responsible for transporting monoamines into vesicles and can be regarded as an indicator of the integrity of striatal DA nerve terminals (Vander Borght *et al.*, 1995; Wilson & Kish, 1996). In correlation with these preclinical data, clinical studies have also reported decreased DAT levels following MA-exposure (McCann *et al.*, 1998; Sekine *et al.*, 2001; Volkow *et al.*, 2001c). Wilson *et al.* (1996) hypothesized that reduced DAT levels could explain the need for escalating dosages and dysphoria observed in humans chronically abusing MA.

The neurotoxic effects of MA can also be due to damage to 5-HT fibres through an unknown mechanism (Nordahl *et al.*, 2003). However, Johnson *et al.* (1987) and Sonsalla *et al.* (1986) postulated that MA-induced DA release is believed to be an intermediate step in this neurodegenerative process, considering that the administration of DA synthesis inhibitors prevents the reported damage and subsequent neurodegeneration of 5-HT neurons (Schmidt *et*

al., 1985; Stone *et al.*, 1986). Another preclinical study in rats reported lowered 5-HT levels in the medial frontal cortex and striatum following the administration of toxic MA doses (Abekawa *et al.*, 1996). Thus, the mechanism by which MA-administration contributes to reduced 5-HT concentrations and -degeneration seems to be complex and may involve other neurotransmitter systems (Callahan *et al.*, 2001).

Acute MA-administration also significantly increases the release of glutamate, which may contribute to the neurotoxic effects induced by MA (Nash & Yamamoto, 1992; Abekawa *et al.*, 1994). Several studies reported an increase in extracellular glutamate levels in the striatum (Wagner *et al.*, 1980; Stephans & Yamamoto, 1994) and hippocampus (Grinspoon & Hedblom, 1975) following the administration of MA due to the activation of D_1 receptors on the presynaptic striatonigral terminals (Rogers *et al.*, 1999). This activation causes an increase in GABA-release in the substantia nigra pars reticulata (Simon *et al.*, 2000b; Nordahl *et al.*, 2003), thereby decreasing the activity of GABA in the nigrothalamic region, resulting in subsequent disinhibition of glutamate in the corticostriatal region (Burns *et al.*, 1967). Moreover, MA-induced damage to GABAergic-functioning in the cortex will lead to schizophrenia-like psychosis (Hsieh *et al.*, 2014). Tata and Yamamoto (2007) reported that over activation of glutamate receptors by MA will result in oxidative stress (discussed later in this chapter, section 2.6), which is believed to substantially contribute to the neurotoxic effects associated with MA-abuse.

Thus, DA, the formation of NO, 5-HT and glutamate play a primary role in MA's ability to produce neurotoxic effects and its consequential neurodegenerative properties.

2.6 Oxidative stress induced by drugs of abuse

A recent study reported that several drugs of abuse induce oxidative stress (Cunha-Oliveira *et al.*, 2013a) and accumulating evidence supports the notion that oxidative stress is implicated in the toxicity induced by several drugs of abuse in the liver, kidneys, heart and of relevance, the brain (Carvalho *et al.*, 2012; Riezzo *et al.*, 2012). The brain is especially vulnerable to oxidative stress due to the abundant presence of polyunsaturated fatty acids, its high oxygen consumption and decreased presence of antioxidant enzymes (Wang *et al.*, 2009). Consequently the ability of drugs of abuse to induce oxidative stress in the CNS may substantially contribute to their addiction potential (Leshner, 1997). Moreover, psychotic disorders are widely regarded to underscore a disturbance in redox balance (Brand *et al.*, 2015) while antioxidants like N-acetyl cysteine (NAC) may have antipsychotic-like properties in both humans ((reviewed in Dean *et al.* (2011)) and animals (Möller *et al.*, 2013a; Swanepoel *et al.*, 2018). The metabolic byproduct of EFV, 8-OH-EFV, has been shown to act as a potent pro-

oxidant (Tovar-y-Romo *et al.*, 2012; Decloedt & Maartens, 2013; Dalwadi *et al.*, 2018), so that the induction of oxidative stress may be one of the primary mechanisms by which EFV exerts its purported psychogenic and neurotoxic effects. In fact several studies have reported that EFV exposure in human cell lines induces an oxidative-inflammatory response (Apostolova *et al.*, 2010; Díaz-Delfín *et al.*, 2011; Weiß *et al.*, 2016). Weiß *et al.* (2016) also reported that pharmacologically relevant concentrations of EFV lead to the production of reactive oxygen species (ROS) in various human tissue cell types. A clinical study supports this finding, with evidence that pre-treatment with antioxidants partially reverse the toxic effects of EFV, suggesting the involvement of ROS in mediating these effects (Apostolova *et al.*, 2010). In correlation with this, a preclinical study recently conducted in our laboratory also established that sub-chronic exposure to the most rewarding dose (5 mg/kg) of EFV increased ROS in rats (Möller *et al.*, 2018). The development of drug-induced oxidative stress may derive from direct or indirect effects (Cunha-Oliveira *et al.*, 2013b), including the overproduction of ROS, mitochondrial dysfunction and the oxidative metabolism of monoamines (Cunha-Oliveira *et al.*, 2013a).

ROS are regarded as normal products of aerobic metabolism (Sies, 1997) and include peroxyl (ROO·), hydroxyl (·OH), alkoxyl (RO·), superoxide (O_2·) or nitroxyl (NO·) and non-radical entities viz. hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and organic hydroperoxides (ROOH) (Simon *et al.*, 2000a; Brieger *et al.*, 2012). They play an important role in cell signalling, the regulation of cell growth and blood pressure as well as cognitive functioning (Brieger *et al.*, 2012). ROS are also responsible for mediating inflammatory responses. These highly reactive, diffusible entities also form part of the body's inherent antimicrobial defence mechanism and play a vital role in immune function (Simon *et al.*, 2000a). The antimicrobial action of ROS should be very specific to foreign microbes, considering that ROS have the ability to cause substantial, non-specific damage to both DNA and proteins (Simon *et al.*, 2000a) which will lead to serious tissue damage and consequential inflammation (Smith, 1994; Vachier *et al.*, 1994). ROS are generated in a cascade of transitions from one species to another, as depicted in figure 2-5. The human body's inherent defence system against ROS consists of antioxidant enzymes and antioxidant scavengers (Brieger *et al.*, 2012). These defence systems include all forms of protection: prevention, interception and repair (Sies, 1997). Enzymatic (catalase and superoxide dismutase (SOD)) and nonenzymatic (glutathione (GSH) peroxidases) antioxidants (Do *et al.*, 2009) are substances that have the ability to inhibit the oxidation of a substrate (provided they are present in lower concentrations than the oxidisable substrate) (Halliwell, 1989) and evidently prevent tissue damage (Simon *et al.*, 2000a). Non-enzymatic antioxidants (also called preventative antioxidants) inhibit peroxidation by several interactive mechanisms viz. removing oxygen or metal ions that catalyse reactions; scavenging peroxyl, hydroxyl and

alkoxyl radicals or scavenging singlet oxygen (Gutteridge, 1995). Apart from generating ROS, drugs of abuse such as MA, cocaine and heroin either deplete or impair the activity of these antioxidant defence systems resulting in the development of oxidative stress (Cunha-Oliveira *et al.*, 2013a).

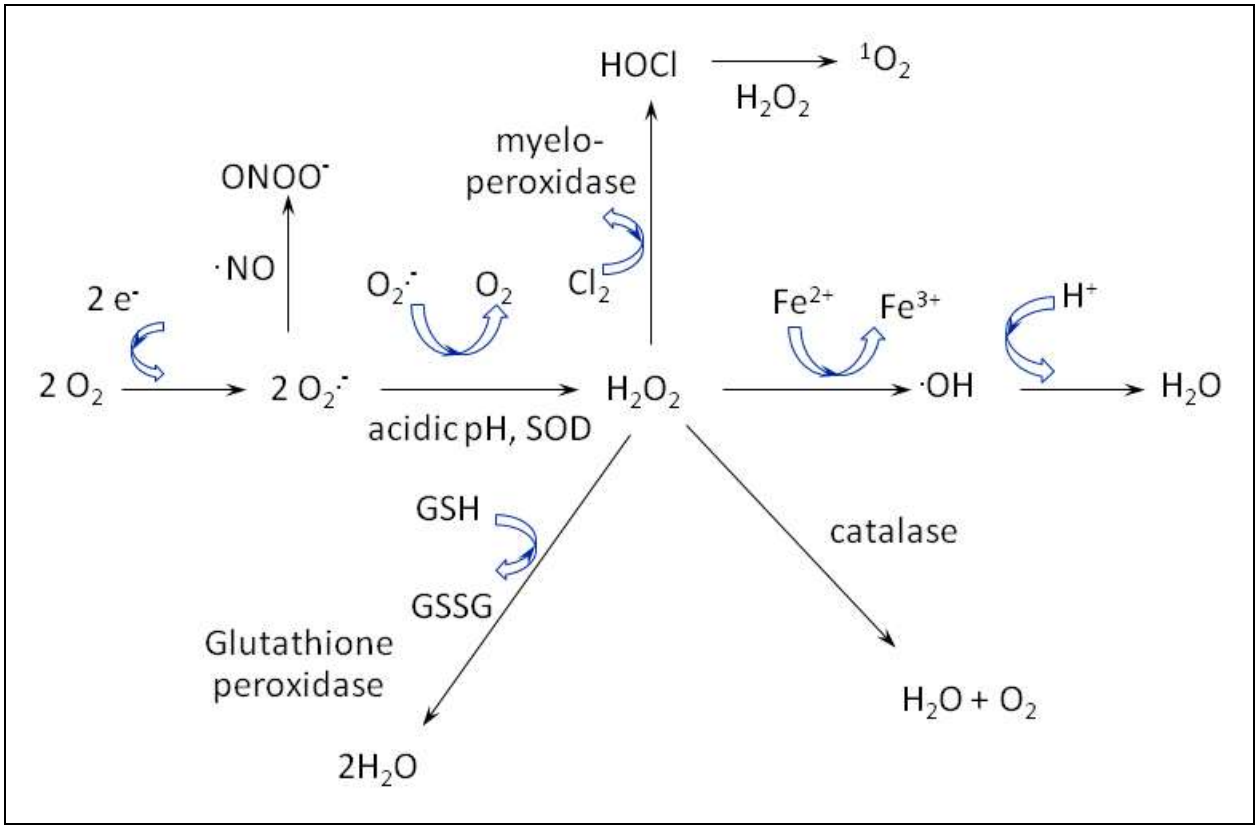


Figure 2-7: ROS generation transition cascade. Notably the unstable superoxide anion ($O_2^{\cdot-}$) rapidly dismutates into hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD), or it reacts with nitric oxide ($^{\cdot}NO$) to produce peroxynitrite ($ONOO^-$) Single oxygen ($^1O_2$) is produced when the H_2O_2 reacts with hypochlorous acid (HOCl). GSH, glutathione; GSSG, glutathione disulfide (Brieger *et al.*, 2012).

Exposure to prolonged high levels of ROS will cause oxidative stress due to the ability of high ROS concentrations to induce irreversible damage to and destruction of proteins, carbohydrates, lipids and nucleic acids (Brieger *et al.*, 2012). Overproduction of ROS and other reactive metabolites occur during the oxidative metabolism of several drugs of abuse (such as the amphetamines and cocaine) (Cunha-Oliveira *et al.*, 2013a) and during certain pathological conditions (Brieger *et al.*, 2012). As previously mentioned, ROS are continuously produced by aerobic metabolism, even under basal conditions (Sies, 1994), and constantly need to be inactivated in order to prevent the occurrence of oxidative stress. The earlier mentioned enzymatic and non-enzymatic antioxidants safely remove ROS, thereby preventing the subsequent development of oxidative stress and associated cell injury and damage to tissues (Gutteridge, 1995).

Sies (1997) defined oxidative stress as an imbalance between oxidants and antioxidant defence systems in favour of oxidants, potentially leading to cell injury. This process precedes or accompanies cell and tissue damage and consequential cell death due to the overproduction of ROS and oxidation of lipids, DNA and proteins (Cunha-Oliveira *et al.*, 2008). Rats exposed to MA present with increased lipid oxidation levels in the PFC, striatum, amygdala and hippocampus leading to hyperlocomotion (da-Rosa *et al.*, 2012). Furthermore, another preclinical study also reported oxidative brain damage due to increased lipid peroxidation levels in the hippocampus of rats exposed to cocaine in utero (Bashkatova *et al.*, 2006). Heroin administration to mice causes oxidation of proteins, DNA and lipids in the brain (Qiusheng *et al.*, 2005). Fourie et al., 2018 established that the most rewarding dosage of EFV (5 mg/kg) significantly increases lipid peroxidation in the frontal cortex and striatum of rats.

A preclinical study established that MA causes oxidative damage by decreasing GSH peroxidases and increasing catalase levels in the brain, kidneys and liver of rats (Koriem *et al.*, 2013). Interestingly, these redox changes and their associated behavioural effects can be reversed by the GSH precursor, NAC (Swanepoel *et al.*, 2018). Cocaine on the other hand, has been found to decrease catalase activity in the PFC and striatum of mice (Macêdo *et al.*, 2005) but to increase SOD levels in the same brain regions in rats (Dietrich *et al.*, 2005). Another preclinical study also reported that lower GSH levels in the hippocampus of rats exposed to cocaine resulted in deficits in cognitive processes such as memory and learning (Muriach *et al.*, 2010). As previously mentioned, the development of oxidative stress induced by drugs of abuse may also derive from the oxidative metabolism of monoamines (Cunha-Oliveira *et al.*, 2013a) and that DA is a key neurotransmitter involved in the neurobiology of drug abuse (Tomkins & Sellers, 2001). DA has neurotoxic effects both *in vivo* (Hastings *et al.*, 1996) and *in vitro* (Graham *et al.*, 1978; McLaughlin *et al.*, 1998). The metabolism of higher DA concentrations (produced by drugs of abuse) at the synaptic cleft by either monoamine oxidase or auto-oxidation will result in the overproduction of ROS, quinones and semiquinones which have neurodegenerative properties (Purohit *et al.*, 2011). Monoamine oxidase is responsible for the intracellular metabolism of DA and the resulting oxidative deamination leads to the formation of the highly toxic 3,4-dihydroxyphenylacetaldehyde which is further metabolised by aldehyde dehydrogenase to form DOPAC and the pro-oxidant H_2O_2 (Marchitti *et al.*, 2007). A previous preclinical study conducted by Campolongo *et al.* (2007) reported increased monoamine oxidase B expression as a result of Δ^9-THC administration, probably as a result of a reactive response in the presence of elevated monoamine levels and neurotransmission in the CNS. Furthermore, the accumulation of synaptic DA will diffuse and bind to adjacent DA receptors, also causing oxidative stress due to the production of pro-inflammatory cytokines and

chemokines (Kuhn *et al.*, 2006). This neuroinflammatory response will be discussed in more detail in section 2.7.

2.7 Inflammatory response of drugs of abuse

It has been proposed that psychostimulant drugs of abuse (viz. cocaine, MDMA and MA) could in part exert their neurotoxic and other deleterious effects by inducing neuroinflammation (Clark *et al.*, 2013). Neuroinflammation results from several factors including the activation of microglia and astroglia (often referred to as gliosis or 'priming') and the synthesis or release of pro-inflammatory molecules such as cytokines, chemokines (cytokines able to induce chemotaxis) and NO (Minagar *et al.*, 2002; O'Callaghan & Sriram, 2005; O'Callaghan *et al.*, 2008; Clark *et al.*, 2013).

2.7.1 Brain glia activation

The brain glia have dual protective and destructive properties (Minagar *et al.*, 2002). Approximately 10-15% of all CNS cells are microglia that play an important role in mediating the innate immune response to disease and injury within the brain (Streit *et al.*, 1999; Clark *et al.*, 2013). All types of CNS damage (whether evoked by drugs of abuse or other contributing factors such as disease or trauma) will result in the activation of brain glia (microglia and astroglia) (O'Callaghan & Sriram, 2005; O'Callaghan *et al.*, 2008). Such activation is linked to the production and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), chemokines, complement factors and other substances that are responsible for initiating and modulating the innate immune response (Minagar *et al.*, 2002; Clark *et al.*, 2013). Escubedo *et al.* (1998) reported activation of brain glia and decreased dopaminergic terminals in the striatum following chronic MA administration to rats. LaVoie *et al.* (2004) further elaborated that the MA-induced activation of microglia precedes injury to striatal dopaminergic neurons. More recently, agents that target the above-mentioned microglial processes, such as rolipram and ibudalast, have realised new potential in treating MA addiction (Iyo *et al.*, 1995; Beardsley *et al.*, 2010).

2.7.2 Pro-inflammatory cytokines and their relation to neuroinflammation

Cytokines are produced in a cascade reaction prompted by the action of macrophages, endothelial cells, Schwann cells and mast cells during pathological processes such as infection, injury and acute inflammation (Zhang & An, 2007). Moreover, cytokines mediate the physiologic and metabolic response to stress and injury (Wang *et al.*, 1999), and can be divided

into pro-inflammatory (e.g. IL-1β, IL-6 and TNF-α)) and anti-inflammatory (e.g. interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-4 (IL-4) and interleukin-13 (IL-13) cytokines (Zhang & An, 2007).

The release of pro-inflammatory cytokines by the above will induce inflammatory reactions (Tracey *et al.*, 1987; Dinarello, 1994; Wang & Tracey, 1999; Minagar *et al.*, 2002) but also during the process of gliosis in response to CNS injury (O'Callaghan & Sriram, 2005). IL-1β, IL-6 and TNF-α are the pro-inflammatory cytokines mostly implicated in initiating and mediating the innate immune response and promoting neuroinflammation (Clark *et al.*, 2013). Drugs of abuse such as MA causes over activation of microglial cells, which leads to the release of pro-inflammatory cytokines and neuroinflammation (Thomas *et al.*, 2004; Cadet *et al.*, 2007). Supporting this, a preclinical study performed in mice found that acute high doses of MA increased messenger ribonucleic acid (mRNA) expression of IL-6 and TNF-α (which promotes neuroinflammation) in the hippocampus and striatum (Gonçalves *et al.*, 2008). These inflammatory reactions can be inhibited/controlled by immunoregulatory anti-inflammatory cytokines (such as IL-10) (Cassatella *et al.*, 1993), due to its potent anti-inflammatory properties (Zhang & An, 2007).

2.7.3 Implication of neuroinflammation: Altered tryptophan metabolism

Tryptophan metabolism entails the conversion of tryptophan into 5-HT, by the enzyme tryptophan-5-hydroxylase, or into kynurenine by the two principle haemdependant enzymes tryptophan-2,3-dioxygenase (TDO) in the liver or indoleamine-2,3-dioxygenase (IDO) in the lungs, CNS and placenta (Möller *et al.*, 2015). Oxidative stress (as well as increased TNF-α and decreased IL-10) induces a pro-inflammatory state that shifts tryptophan-metabolism towards the kynurenine pathway by activating TDO or IDO (see figure 2-6) (Möller *et al.*, 2015). TDO is only responsible for the oxidative metabolism of tryptophan whilst IDO also metabolizes 5-HT and melatonin (Stone & Darlington, 2002).

The metabolism of kynurenine by kynurenine-aminotransferases (KAT) (Han *et al.*, 2010) produces kynurenic acid (KYNA) (see figure 2-6), which has neuroprotective effects due to its endogenous, non-competitive *N*-methyl-*D*-aspartate (NMDA) antagonist properties at the facilitatory glycine site on the NMDA receptor ion channel (Connor *et al.*, 2008; Han *et al.*, 2010). However, kynurenine can also be metabolised by kynurenine-3-monooxygenase (KMO) to 3-hydroxykynurenine, anthranilic acid (AA), 3-hydroxyanthranilic acid and the excitotoxin,

quinolinic acid (QA) (see figure 2-6) (Connor *et al.*, 2008). QA acts as a NMDA receptor agonist with consequential neurodegenerative properties (Schwarcz, 2004; Möller *et al.*, 2015) that evokes cell death (apoptosis) of human astrocytes (Guillemin *et al.*, 2005). KYNA plays an important role in protecting the astrocytes against the neurodegenerative and excitotoxic effects of QA (Kim & Choi, 1987; Stone & Darlington, 2002). The KYNA: kynurenine ratio is therefore also known as the neuroprotective ratio which is indicative of the neuroprotective-neurodegenerative balance in the brain (Myint *et al.*, 2007; Myint *et al.*, 2011).

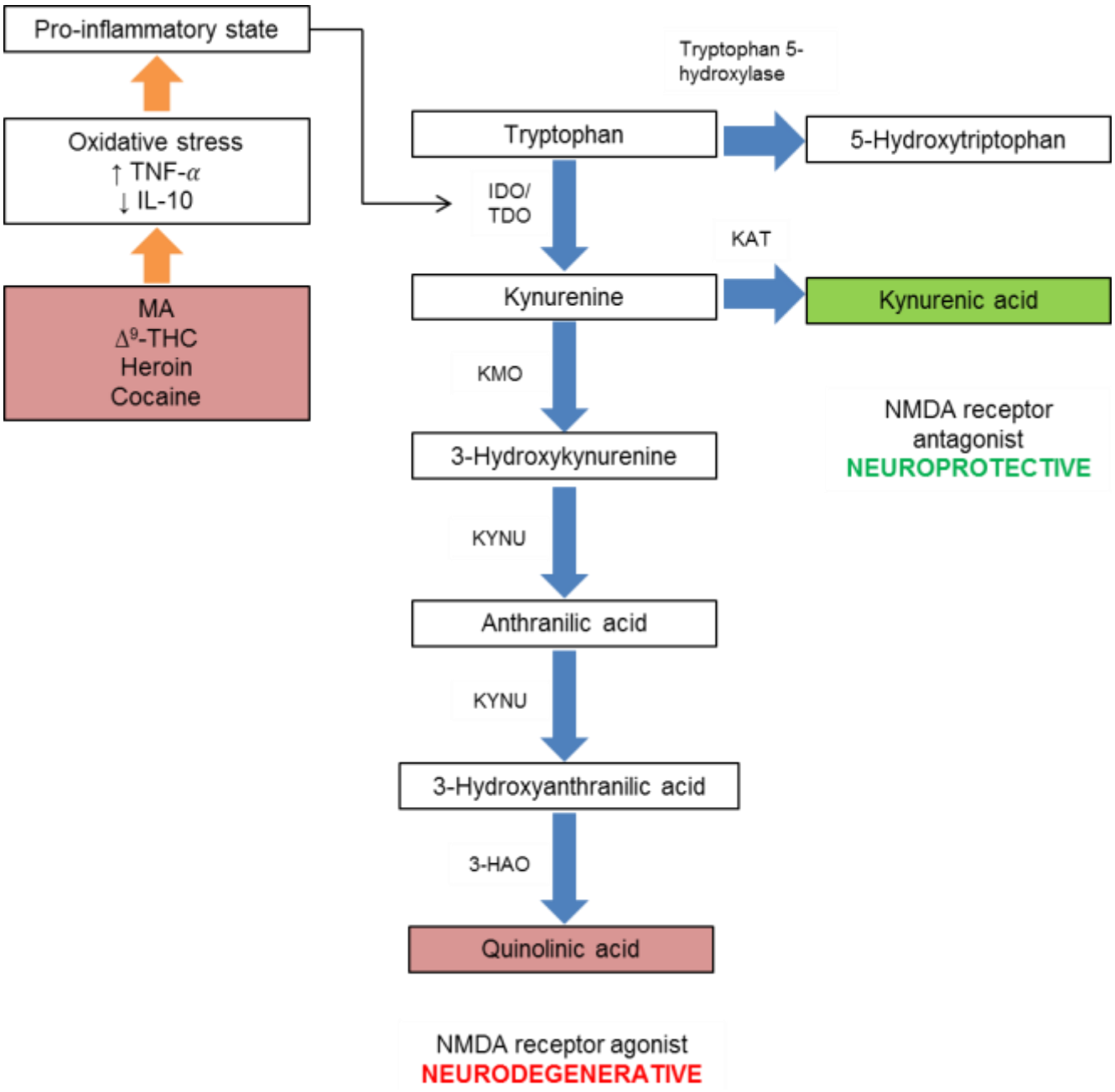


Figure 2-8: Simplified diagram of tryptophan metabolism via the kynurenine pathway and its activation via an inflammatory-oxidative stress response, as induced by known drug of abuse. See text for more details. *TNF-α, Tumor necrosis factor alpha; IL-10, Interleukin 10; IDO, Indoleamine-2,3-dioxygenase; TDO, Tryptophan-2,3-dioxygenase; KMO, Kynurenine-3-monooxygenase; KAT, Kynurenine-aminotransferases; KYNU, Kynureninase; 3-HAO, 3-Hydroxyanthranilate oxidase; MA, Methamphetamine; Δ9-THC, Δ9-Tetrahydrocannabinol; NMDA, N-methyl-D-*

Earlier work has established the connection between disturbances in kynurenine metabolism and psychiatric illness, in particular depression (Myint *et al.*, 2012), schizophrenia and psychosis (Muller *et al.*, 2011; Erhardt *et al.*, 2017). Importantly, animal model work has confirmed the kynurenine pathway to be a definite target for antipsychotic response (Möller *et al.*, 2012; Möller *et al.*, 2013a).

Several preclinical studies have established that MA causes over-activation of microglial cells, which leads to the release of pro-inflammatory cytokines and an increased inflammatory response (Thomas *et al.*, 2004; Cadet *et al.*, 2007). Moreover, as previously mentioned in section 2.6, EFV exposure in human cell lines also induces an oxidative-inflammatory response (Apostolova *et al.*, 2010; Díaz-Delfín *et al.*, 2011; Weiß *et al.*, 2016), which could in part be attributed to the ability of EFV to promote the release of pro-inflammatory cytokines (such as IL-6, IL-8 and monocyte chemoattractant protein-1) and other molecules involved in inflammatory processes (hepatocyte growth factor and plasminogen activator inhibitor type 1). In support of this, O'Mahony *et al.* (2005) reported that EFV-exposed rats had increased plasma pro-inflammatory (IL-1β and TNF-α) cytokines, which were related to depressive-like behaviours and a greater susceptibility to stress. The lack of data describing the effects of drugs of abuse on the kynurenine pathway is a limitation in current research. However two preclinical studies observed that EFV exposure in rats caused a significant decrease in TDO activity, which might relate to an enhancing effect on 5-HT levels (Zheve, 2007; Cavalcante *et al.*, 2010). These studies indicate a clear pro-inflammatory response and emphasize the possible role of the kynurenine pathway in inducing the neuropsychiatric effects observed after EFV exposure (Calvalcante et al., 2010).

In conclusion, increased 5-HT in the striatum and hippocampus as well as increased DA and NE in the limbic system correlate with anxiogenic behaviour (Morilak *et al.*, 2005; Rex *et al.*, 2005; Leranth & Hajszan, 2007; Watt *et al.*, 2009). Moreover, increased striatal NE have been implicated in psychosis (Brand *et al.*, 2015). Abnormalities in 5-HT neurotransmission have also been linked to the neurobiological origin of psychotic states (Geyer & Vollenweider, 2008), considering that serotonergic hallucinogens (such as LSD and psilocybin) reduce PPI in rats (Geyer, 1998) and in humans (Vollenweider *et al.*, 2007). Thus, drug-induced elevations in monoamine levels, especially DA, will evidently lead to oxidative stress (Cunha-Oliveira *et al.*, 2013a) and neuroinflammation (Kuhn *et al.*, 2006). Since altered redox has been related to regional brain changes (Möller *et al.*, 2013a; Möller *et al.*, 2013b), it is evident that such changes will drive alterations in behaviour. Supporting this, previous preclinical studies

reported altered DA and 5-HT levels in the limbic brain regions due to altered redox and a pro-inflammatory state (Brand *et al.*, 2015; Möller *et al.*, 2015). These neurochemical alterations significantly contributed to mood-altering, psychogenic and anxiety-related behavioural manifestations (Brand *et al.*, 2015; Möller *et al.*, 2015). Interestingly, Dalwadi *et al.* (2018) reported that EFV and / or its metabolites (specifically, 8-OH-EFV) may possibly cause disturbances in the kynurenine pathway due increased pro-inflammatory cytokines (see figure 2.8) and that these disturbances could be responsible for some of the reported neurobehavioral alterations induced by EFV.

2.8 Preclinical addiction research

Drug abuse is widely perceived as a disease humans inflict upon themselves, therefore great controversy exists regarding the use of animal models in the field of addiction biology (Lynch *et al.*, 2010). However, exceptionally well developed and validated animal models on substance abuse and addiction are considered to be the best available models for researching neuropsychiatric disorders (O'Brien & Gardner, 2005; Kalivas *et al.*, 2006). These animal models have been invaluable for researchers investigating the effects of drugs of abuse on specific neurochemical alterations, neuroanatomical circuitry, neurophysiological functions and behavioural processes underlying drug addiction (Frascella *et al.*, 2011), since they induce the same motivationally driven behaviour seen in human addiction (Kalivas *et al.*, 2006). Moreover, self-administration procedures established that drugs of abuse have reinforcing properties in animals (Lynch *et al.*, 2010). Griffiths *et al.* (1979) further emphasized the particular importance of these animal models by reporting that they can accurately predict drug preferences in humans. Furthermore, these animal models enable researchers to obtain neuroadaptive clues that would practically be impossible to procure from human studies (Frascella *et al.*, 2011). The use of laboratory animals in a controlled laboratory setting provides researchers with a meticulous method to precisely control and manipulate several aspects of drug addiction (viz. the environmental setting and drug exposure) (Belin-Rauscent & Belin, 2012). These animal models also provide a rigorous means to accurately assess behaviour and cognition prior to drug exposure (Belin-Rauscent & Belin, 2012). It is therefore clear that this degree of control can only be practically feasible in animals. However, animal studies in drugs of abuse present with some limitations as they can never fully reproduce the complex background setting as to why people abuse drugs (Belin-Rauscent & Belin, 2012), which could possibly explain why drug addiction remains one of the most serious neuropsychiatric disorders resulting in catastrophic personal and social costs (Kalivas *et al.*, 2006).

The predominate neuropsychiatric adverse effects of the most drugs of abuse, especially Δ^9-THC, are anxiety (at relative high dosages) (Crippa *et al.*, 2009) and schizophrenia-like

psychosis (Ames, 1958; Talbott & Teague, 1969; Chopra & Smith, 1974; Gururajan *et al.*, 2012). Behavioural tests, such as the SIT, the EPM and the PPI test are commonly conducted in animals to study these behavioural effects of drugs of abuse and will now be discussed in further detail.

2.8.1 Social interaction test

The SIT is an extensively validated behavioural test that can be used to assess deficits in the instinctive social interactive and self-directed (such as self-grooming) behaviours in rat pairs (File & Seth, 2003). The rats are exposed to an open field arena (Ferdman *et al.*, 2007) and allowed to interact with each other and with the environment. Rats with a similar genetic background exposed to the same prior experiences and drug administration should be used considering that the behavioural profile of one rat can affect that of the other rat (File & Seth, 2003; Haller *et al.*, 2004). An increase in social interactive behaviour (such as rearing, anogenital sniffing, approaching, grooming and following the rat partner) (Gonzalez *et al.*, 1996) and decrease in self-directed behaviour are indicative of decreased social-anxiety whilst a decrease in social interactive behaviour (immobility when the rat partner is approaching and total avoidance of the rat partner) and elevated self-grooming suggest an anxiogenic effect in a social setting (File & Seth, 2003; Möller *et al.*, 2011). Other preclinical studies on drugs of abuse evaluating SIT concluded that LSD (Marona-Lewicka *et al.*, 2011), amphetamine (File & Hyde, 1979) and cannabinoids (such as 5 mg/kg Δ^9-THC) (Cutler & Mackintosh, 1984) significantly decreased social interaction and promoted social withdrawal. Social withdrawal is one of the primary negative symptoms observed in psychosis (Sams-Dodd *et al.*, 1997), suggesting that drugs of abuse could contribute to the induction of a psychotic state together with social withdrawal. However, a pre-clinical study by (Thiel *et al.*, 2008) indicated that cocaine enhanced social behaviour, suggesting that social interaction during the use of cocaine (and possibly other drugs of abuse) might have a rewarding effect. In their review Blanco-Gandía *et al.* (2015) comprehensively describe that drugs of abuse such as MDMA, psychostimulants (cocaine, amphetamine and MA) and cannabinoids (such as Δ^9-THC) decrease social interaction whilst opiates are known to increase social behaviour.

2.8.2 The elevated plus maze

EPM is a very useful and well validated (Pellow *et al.*, 1985) behavioural test for evaluating fear and anxiety responses in rodents (Hogg, 1996) exposed to drugs of abuse. The plus-shaped maze has two opposite open arms and two closed arms and is elevated above the ground (Fischer *et al.*, 2012). Rats have a proclivity towards the darker, enclosed arms of the maze and will generally avoid the open arms due to a fear of heights or open spaces (Walf & Frye, 2007). The plasma corticosterone concentration of rats will increase when they access the

open arms of the maze and they will exhibit adaptive behavioural changes such as increased freezing and the production of faecal matter indicative of increased anxiety (Pellow *et al.*, 1985; Hogg, 1996). Several studies have reported that the administration of drugs of abuse such as cannabinoids and MDMA will induce anxiety-like behaviour in rats and increase their natural aversion towards the open arms of the maze (Onaivi *et al.*, 1990; Onaivi *et al.*, 1995; Sumnall *et al.*, 2004; Rutkowska *et al.*, 2006).

A recent study also investigated the effects of EFV exposure on the EPM behavioural test and found that sub-chronic EFV (50 mg/kg) caused a significant decrease in the time spent in the open arms of the maze and in the % of open arm entries, suggesting an anxiogenic-like effect (Cavalcante *et al.*, 2017).

2.8.3 The pre-pulse inhibition test

Another useful behavioural test for evaluating the psychoactive profile of drugs of abuse is sensorimotor gating, evaluated in the PPI test (Boucher *et al.*, 2007; Arai *et al.*, 2008; Todd *et al.*, 2017). PPI of the startle reflex is an acoustic neurological behavioural test that uses a weak sensory prestimulus (prepulse) to diminish the startle response (indicated by contraction of skeletal and facial muscles in humans (Braff *et al.*, 2001)) evoked by a secondary stronger stimulus presented several milliseconds (30-100 ms) after the prepulse (Graham, 1975; Arai *et al.*, 2008). PPI can therefore be used to measure sensorimotor gating. Animal model studies of sensorimotor gating enable us to understand the functional importance of attentional abnormalities and DA alterations in patients with psychosis (Braff & Geyer, 1990). Moreover, Boucher *et al.* (2007) revealed that Δ^9-THC exposure in mice, notably increases PPI, whilst Arai *et al.* (2008) observed that both acute and chronic MA exposure in rats significantly reduced PPI, possibly by stimulating DA neurotransmission at the synaptic cleft Previous studies in our laboratory also indicated a significant deficit in PPI in rats exposed to MA (Strauss *et al.*, 2014; Swanepoel *et al.*, 2017). Another study also reported that the direct or indirect stimulation of DA receptors causes significant deficits in PPI (Braff *et al.*, 2001). However, the effects of EFV on PPI have as yet not been assessed.

2.9 Synopsis

The bio-behavioural profile evoked by drugs of abuse significantly contributes to their addiction - and abuse potential due to the development of drug dependence. The NNRTI, EFV is the ART associated with the most neuropsychiatric manifestations (Cespedes & Aberg, 2006) including (but not limited to): anxiety, depression and acute psychosis (Gatch *et al.*, 2013). The psychoactive profile of EFV most likely encouraged its widely reported recreational use in a potent multi-drug cocktail termed “Nyaope” or “Whoonga” (Marwaha, 2008; Sciutto, 2009; Hull, 2010; Cullinan, 2011; Fihlani, 2011) , which allegedly also contains other known drugs of abuse such as marijuana (Hull, 2010). Several preclinical studies elaborated on the abuse and addiction potential of EFV. Gatch *et al.* (2013) reported that the pre-dominate behavioural profile of EFV is in line with that of LSD, also mediated by weak partial agonism on the 5-HT_{2A} receptor subtype. Moreover, our laboratory recently established that sub-chronic exposure to EFV (5 mg/kg) produced significant rewarding effects in the CPP paradigm (Möller *et al.*, 2018). However, very limited scientific data exploring the behavioural profile of EFV within an addiction setting exists, with only one preclinical study reporting that sub-chronic EFV exposure induces depressogenic (at 25 mg/kg and 50 mg/kg) and anxiogenic (at 50 mg/kg) behaviour in rats (Cavalcante *et al.*, 2017). Behavioural tests such as the SIT, EPM and PPI of the acoustic startle reflex are invaluable in evaluating the psychological profile of a rewarding dose of EFV (5 mg/kg) with regards to social interactive behaviour (File & Seth, 2003), anxiety-like behaviour (Hogg, 1996) and sensorimotor domains (Boucher *et al.*, 2007; Arai *et al.*, 2008; Todd *et al.*, 2017) respectively.

The underlying neuro-pharmacological mechanisms by which these drugs produce their characteristic behavioural profile potentially involve a wide range of neurotransmitter systems (especially those involving DA, 5-HT and NA), central hormone imbalances (particularly those involving OT) and disturbances in the immune-inflammatory response. Möller *et al.* (2018) recently reported that sub-chronic exposure to EFV increases cortico-striatal levels of DA and 5-HT as well as NA levels in the striatum. The effects of drugs of abuse on the ‘pro-social’ hypothalamic peptide hormone and neuromodulator, OT, can significantly influence its behavioural profile with regards to social interaction (Zhou *et al.*, 2008; McRae-Clark *et al.*, 2013). Elevated OT levels are generally associated with increased social interactive behaviour (Thompson *et al.*, 2007), whilst decreases in OT are known to promote social withdrawal (Butovsky *et al.*, 2006; O'Shea *et al.*, 2006). Drugs of abuse can exert their neurotoxic and neurodegenerative effects possibly by inducing a pro-inflammatory state in the brain (Clark *et al.*, 2013). This pro-inflammatory state could be the result of increased pro-inflammatory (TNF-

α) - and decreased anti-inflammatory (IL-10) cytokines (Möller *et al.*, 2013a) as well as oxidative stress (Cunha-Oliveira *et al.*, 2013a). Möller *et al.* (2018) also reported altered redox states with peripheral oxidative stress in rats exposed to sub-chronic EFV (5 mg/kg). The pro-inflammatory state will evidently shift tryptophan metabolism via the kynurenine pathway and lead to increased production of the excitotoxic, neurodegenerative metabolites such as QA and decreased production of the neuroprotective metabolite, KYNA (Möller *et al.*, 2015). This is of relevance considering that disturbances in kynurenine metabolism have been implicated in various psychiatric illnesses (Muller *et al.*, 2011; Myint *et al.*, 2012; Erhardt *et al.*, 2017).

Therefore, this study will utilize the SIT, EPM and PPI behavioural tests as well as peripheral and neurochemical analysis to determine the bio-behavioural profile of a rewarding dose of EFV alone or in combination with Δ^9-THC (the psychological component of marijuana (Grinspoon & Bakalar, 1997)). Moreover, this study will further investigate the possible neuro-pharmacological mechanisms mediating these behavioural effects.

2.10 References

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CHAPTER 3

ARTICLE

This chapter serves as a concept article for submission to *Brain, Behavior and Immunity*, a peer-reviewed scientific journal, with an impact factor of 6.306 (2017). The article was prepared according to the authors' guidelines (see https://www.elsevier.com/journals/brain-behavior-and-immunity/0889-1591/guide-for-authors).

The title page of the article includes the title, contributing authors, the affiliation for each author, the abstract and keywords. The outline of the article is as follows: Introduction, Materials and methods, Results, Discussion, Conclusion, Acknowledgements, Funding and References.

The format of this concept article has been adapted for for the benefit of the reader, for this reason, all figures have been inserted in the text. Moreover, the heading, page and figure numbers, format and layout of this article will align with the dissertation. Before submission of the article, these changes will be amended.

N. Muller assisted with the design of the study, conducted the behavioural, neurochemical and peripheral analysis and created the first draft of this dissertation. With the help of M. Möller-Wolmarans, she also undertook the statistical analysis. B.H. Harvey also advised on the study design and statistical analysis. M. Möller-Wolmarans and B.H. Harvey conceptualized the idea for this study and the study design as well as supervised the study, assisted in the interpretation of data and finalized the dissertation for publication.

All co-authors granted their permission for the article to be submitted for the purpose of the M.Sc. degree (Addendum C).

Title

Exposure to efavirenz alone or in combination with Δ^9-tetrahydrocannabinol induces selected bio-behavioural changes that define its abuse potential

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Abstract

The central nervous system (CNS) is involved in mediating the neuropsychiatric side effects produced by efavirenz (EFV). The illicit use of EFV in a cannabis-containing cocktail termed "Nyaope" has been described in South Africa. Investigation into the CNS involvement and psychological profile of EFV could provide insight on the reported abuse and addictive profile of this drug. The bio-behavioural profile of a rewarding dose of EFV (5 mg/kg/day), Δ^9-tetrahydrocannabinol (Δ^9-THC) (0.75 mg/kg/day) and EFV + Δ^9-THC (above dosages) × 17 days of alternate drug-vehicle exposure was evaluated. Behavioural analysis included the social interaction test (SIT), elevated plus maze (EPM) and pre-pulse inhibition (PPI). Hippocampal oxytocin (OT) levels in response to the above-mentioned exposure groups were also investigated. The presence of a pro-inflammatory state was considered through analysis of plasma pro- vs. anti-inflammatory cytokines, viz. tumour necrosis factor alpha (TNF-α) and interleukin-10 (IL-10), and tryptophan-kynurenine metabolites. EFV produced deficits in social behaviour, increased anxiety and plasma TNF-α levels and suppressed PPI. Δ^9-THC induced deficits in social interaction, decreased PPI and induced anxiolytic behaviour as well as a reduction in plasma IL-10 concentrations. EFV + Δ^9-THC promoted social behaviour and increased hippocampal OT levels, while also reducing PPI. All the exposure groups shifted tryptophan metabolism towards the kynurenine pathway and decreased the neuroprotective ratio (kynurenic acid: kynurenine). Concluding, sub-chronic exposure to a rewarding dose of EFV induces deficits in social interaction, anxiogenic- and psychotogenic-like behaviour and a pro-inflammatory state. While Δ^9-THC induces anxiolytic behaviour and deficits in the anti-inflammatory response, EFV is anxiogenic and pro-inflammatory. EFV + Δ^9-THC increased pro-social and psychotogenic-like behaviour. Thus, the behavioural manifestations and underlying peripheral and neurochemical attributes may vary dependent on the composition of "Nyaope", especially the contents of EFV + Δ^9-THC.

Keywords: efavirenz; Δ^9-tetrahydrocannabinol; Nyaope; social interaction; anxiety; pre-pulse inhibition; tryptophan-kynurenine pathway; tumor necrosis factor alpha; interleukin-10; oxytocin

3.1 Introduction

The non-nucleoside reverse-transcriptase inhibitor (NNRTI), efavirenz ((4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one) (EFV), in combination with two non-thymidine nucleoside reverse transcriptase inhibitors (NRTI's), is employed as first-line treatment of the human immunodeficiency virus type-1 (HIV-1) (Riddler *et al.*, 2008) (WHO, 2010). Antiretroviral therapy (ART) regimens containing EFV are preferred to combinations of two or three nucleoside analogues as well as the combination of two nucleoside analogues and the NNRTI, nevirapine (see (Arribas, 2003) for review). EFV readily crosses the blood brain barrier due to its lipophilic physiochemical properties and therefore has sufficient penetration into the central nervous system (CNS) (Abdissa *et al.*, 2015; Dalwadi *et al.*, 2018). These pharmacokinetic attributes are important since EFV is known to produce a wide range of neuropsychiatric symptoms (Marzolini *et al.*, 2001; Lochet *et al.*, 2003; Raines *et al.*, 2005; Cespedes & Aberg, 2006; Gatch *et al.*, 2013), including anxiety, hallucinations, paranoia and acute psychosis (Dalwadi *et al.*, 2018). These psychological effects have been suggested to underlie increasing reports of the recreational use and possible addictive profile of EFV (see Dalwadi *et al.* (2018) for review). Since 2008, several media reports described the recreational use of EFV in a drug cocktail containing marijuana (with Δ^9-tetrahydrocannabinol (Δ^9-THC) being the most prominent psychological component), commonly known as "Nyaope" or "Whoonga" (Marwaha, 2008; Hull, 2010; Cullinan, 2011; Fihlani, 2011). The "Nyaope"-cocktail is either smoked or intravenously injected (Hull, 2010; Tshipe, 2017) to produce a psychological behavioural profile that promotes its abuse-potential eventually leading to drug seeking behaviour.

Drug abuse is linked to numerous symptoms such as anxiety, depression and psychosis (reviewed in (Heimer, 2003; Gururajan *et al.*, 2012). Psychotic manifestations are causally linked to deficits in sensorimotor gating, a neurological filtering process whereby insignificant or excessive stimuli are either screened or removed from consciousness (Braff & Geyer, 1990; Arai *et al.*, 2008). Preclinical studies have also reported deficits in sensorimotor gating as a consequence of exposure to known drugs of abuse (Boucher *et al.*, 2007; Arai *et al.*, 2008; Todd *et al.*, 2017). One such study recently confirmed a dose dependent addictive-like profile for EFV, with sub-chronic and sub-acute EFV (5 mg/kg) exposure- producing a rewarding effect in the conditioned place preference (CPP) paradigm (Möller *et al.*, 2018). Another preclinical study has described the predominate behavioural profile of EFV to be very similar to that of lysergic acid diethylamide (LSD), a well-known drug of abuse (Gatch *et al.*, 2013). Furthermore, sub-chronic exposure to EFV (25 or 50 mg/kg) has been found to induce anxiety and depressive-like behaviour in rats (Cavalcante *et al.*, 2017). The above-mentioned behavioural alterations following drug abuse in general have been attributed to drug-induced immune-neurochemical alterations such as increased pro-inflammatory cytokines (Thomas *et al.*, 2004; O'Mahony *et al.*,

2005a; Cadet *et al.*, 2007), altered tryptophan metabolism via the kynurenine pathway (Zheve, 2007; Cavalcante *et al.*, 2010) and increased oxidative stress (Cunha-Oliveira *et al.*, 2013).

Inflammatory responses shift tryptophan metabolism in favour of the kynurenine pathway (Möller *et al.*, 2015), by activating tryptophan-2,3-dioxygenase (TDO) or indoleamine-2,3-dioxygenase (IDO) which catabolizes tryptophan to kynurenine (Möller *et al.*, 2015). Kynurenine can be metabolised by kynurenine-aminotransferases (KAT) (Han *et al.*, 2010) to produced kynurenic acid (KYNA), a non-competitive *N*-methyl-*D*-aspartate (NMDA) antagonist with neuroprotective properties (Connor *et al.*, 2008; Han *et al.*, 2010; Möller *et al.*, 2012a; Möller *et al.*, 2015). Kynurenine can also be metabolised by kynurenine-3-monooxygenase (KMO), which leads to the production of 3-hydroxykynurenine, anthranilic acid (AA) and quinolinic acid (QA) (Connor *et al.*, 2008; Möller *et al.*, 2012a). QA is a NMDA agonist with neurotoxic properties (Schwarcz, 2004; Möller *et al.*, 2015). Indeed, sub-chronic EFV exposure produces profound alterations in redox states with increased peripheral oxidative stress (Möller *et al.*, 2018). However, the effects of sub-chronic alternate day exposure to a rewarding dose of EFV on plasma inflammatory markers and tryptophan metabolism have not yet been addressed.

Most drugs of abuse are known to cause significant alterations to several neurotransmitters, especially the monoamines, and in several brain regions (Gibbs & Summers, 2002; Müller *et al.*, 2007). These actions contribute significantly to their characteristic psycho-behavioural profiles. Recently, it has been established that sub-chronic exposure to the most rewarding dose of EFV (5 mg/kg) significantly elevates cortico-striatal levels of dopamine (DA) and serotonin (5-HT) and increases striatal levels of noradrenaline (NE) (Möller *et al.*, 2018). Contrariwise, sub-chronic, but not acute, EFV (25 mg/kg or 50 mg/kg) was found to decrease striatal levels of DA, 5-HT and NE (Cavalcante *et al.*, 2017). These contradictory findings with respect to sub-chronic EFV exposure could be attributed to the dose-dependent rewarding and addictive profile of EFV, since higher EFV doses (10 mg/kg and 20 mg/kg) appear to be more aversive (Möller *et al.*, 2018).

A central disturbance in drug abuse involves deficits in social behaviours, presenting predominantly as social withdrawal, but may vary depending on the drug dose, route of administration as well as environmental and genetic predisposition (Blanco-Gandía *et al.*, 2015). Oxytocin (OT) is a nine-amino acid peptide hormone and neuromodulator synthesized in the hypothalamus that is critical in mediating social behaviour in humans and other animals (Sofroniew, 1983; Swanson & Sawchenko, 1983; McGregor *et al.*, 2008). Alterations in OT levels have been suggested to underlie the behavioural profile evoked by drugs of abuse, especially those of a social nature (see McGregor *et al.* (2008) for review). OT neurons project from the hypothalamus to brain regions involved in the modulation of anxiety-like behaviours, in particular the ventral tegmental area, main olfactory bulb, amygdala and medial preoptic area of the

hypothalamus (McGregor *et al.*, 2008; Dabrowska *et al.*, 2011; Rutherford *et al.*, 2011; Knobloch *et al.*, 2012), to produce a range of 'pro-social' and anxiolytic effects (McRae-Clark *et al.*, 2013). Several preclinical studies have reported that drugs of abuse that increase OT levels, also increase social interaction (Kovács *et al.*, 1998; Morley & McGregor, 2000; Thompson *et al.*, 2007), whilst the contrary is true for drugs of abuse that decreases OT levels (Sarnyai *et al.*, 1992; Johns *et al.*, 1997; Butovsky *et al.*, 2006). These opposing actions on the oxytocinergic system could explain the behavioural differences observed between different drugs of abuse. In fact, said actions may contribute to the underlying neuro-behavioural profile induced by EFV abuse, especially when used in combination with other drugs of abuse such as marijuana (e.g. "Nyaope").

The aim of this study was therefore to investigate the effects of EFV, Δ^9-THC as well as the combination of EFV + Δ^9-THC in rats on social interactive- and anxiety-like behaviour as well as sensorimotor gating. Moreover, we considered whether such behavioural changes may be related to alterations in plasma pro- and anti-inflammatory cytokines and/or altered tryptophan-kynurenine metabolism.

3.2 Materials and methods

3.2.1 Ethical statement

This study received appropriate ethical approval from the animal research ethics committee (AnimCare) of the North West University (NWU) (NHREC registration number: AREC-130913-051; ethics approval number: NWU-00278-17-A5). The rats were handled in accordance with the code of ethics in research, training and testing of drugs in South Africa. The study was performed and the article presented according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines, as previously described (Kilkenny *et al.*, 2010).

3.2.2 Animals

Healthy, drug- and behavioural test naïve, adolescent (5-7 weeks old), male Sprague-Dawley (SD) rats, weighing between 150 g and 180 g at the beginning of drug exposure (Vivarium, NWU) were used in this study. Animals were randomly allocated (by an experienced animal technologist blind to the study in order to remove experimental bias (Kilkenny *et al.*, 2010)) to 4 exposure groups with each group containing 18 rats. The number of rats per group were based on previous studies performed in our laboratory using 12 rats per group (Toua *et al.*, 2010), however a statistical power analysis indicated that 12-25 rats in each group would be sufficient to obtain statistical significance for this study design. Therefore, a total of 72 rats were used for this study. The rats were housed under identical conditions in the Vivarium (SAVC registration number: FR15/13458; SANAS GLP compliance number: G0019): 3 rats per individual ventilated cage

(230(h) x 380(w) x 380(l) mm) with corncob bedding and environmental enrichment, temperature (22 ± 2 º C), humidity (55 ± 10%), white light (350-400 lux), 12 h light/dark cycle with food (pellets) and water provided ad libitum (Mouton *et al.*, 2016). To ensure the welfare of the rats, they were monitored on a daily basis throughout the study for any signs of distress or discomfort (by making use of study specific North West University Vivarium monitoring sheets).

3.2.3 Study design

This sub-chronic study consisted of 4 exposure groups receiving either vehicle (pharmaceutical grade olive oil), EFV (5 mg/kg/day) (Möller *et al.*, 2018), Δ^9-THC (0.75 mg/kg/day) (Braida *et al.*, 2004) or the combination of EFV + Δ^9-THC (at above dosages) for a period of 17 days (with alternate day drug exposure) (Möller *et al.*, 2018). The rats were subjected to the following behavioural analysis: the social interaction test (SIT) (on day 12 of exposure), the elevated plus maze (EPM) (on day 14 of exposure) and the prepulse inhibition (PPI) of the acoustic startle reflex test (on day 16 of exposure). All behavioural analyses were conducted on vehicle-exposure days to avoid the acute effects of drug-exposure on behaviour. The rats were euthanized (via decapitation) on day 17 of drug exposure, 2 hours after receiving the last dose of drug, whereupon trunk blood was collected and the hippocampus dissected for inflammation and neuroendocrine assays, respectively. The same rats were used to asses behavioural as well as neurochemical alterations to ensure cost-effectiveness and compliance with ethical standards. All experiments and analysis were blinded to the analyst and person conducting the study by making use of an independent party who colour coded the specific exposure groups. The colour of each specific exposure group was only revealed after all the data has been collected (Curtis *et al.*, 2015). This was done in order to avoid all bias, irrespective of the source, thus in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010).

3.2.4 Drugs and drug exposure protocol

EFV and Δ^9-THC are both lipophilic drugs (Sharma *et al.*, 2012; Gaur *et al.*, 2014), therefore pharmaceutical grade olive oil was used as a vehicle for both these drugs and their combination. Vehicle (0.1 ml) was administered intraperitoneally (i.p.) on days of vehicle exposure. Olive oil has no rewarding properties as it does not produce any preference in the CPP test (da Silveira *et al.*, 2014). Moreover, we recently ruled out any anti-oxidant and monoamine effects of this vehicle (Möller *et al.*, 2018). EFV (Aspen Pharmacare, Port Elizabeth, South Africa) was administered as a single daily i.p. dose of 5 mg/kg/0.2ml/day. This dose was based on a previous sub-chronic preclinical study conducted in our laboratory indicating the rewarding effect of this dose in the CPP paradigm (Möller *et al.*, 2018). Δ^9-THC (Toronto Research Chemicals Inc., Toronto, Canada) was administered i.p. at a dose of 0.75 mg/kg/0.2ml/day. This dose was determined from data described in a preclinical study suggesting that Δ^9-THC, in a range of doses between 0.075 mg/kg

- 0.75 mg/kg, produced significant CPP (Braida *et al.*, 2004). The combination of EFV + Δ^9-THC was also dissolved in the described vehicle and administered i.p.. The vehicle and all drugs were administered (08:00 am – 09:00 am; Vivarium, NWU) for a period of 17 days. The sub-chronic exposure alternated between vehicle and drug exposure beginning with drug exposure on day 1 and continuing alternately for a period of 17 days. Thus, EFV, Δ^9-THC and EFV + Δ^9-THC were administered on days 1, 3, 5, 7, 9, 11, 13, 15 and 17 and vehicle on days 2, 4, 6, 8, 10, 12, 14 and 16 according to a previous study conducted in our laboratory (Möller *et al.*, 2018). The control group only received vehicle throughout the 17 days of exposure.

3.2.5 Body weight

The body weight of each rat was determined on post-natal day (PND) 21 and also on every day of drug/vehicle administration to confirm equal development across all the exposure groups over the study period.

3.2.6 Behavioural tests

All behavioural tests were performed during the dark cycle (from 6:00 pm) in several behavioural analysis rooms according to previously validated methods and in accordance with the AnimCare ethical guidelines (Vivarium, NWU).

3.2.6.1 Social interaction test (SIT)

The SIT is used to analyse self- and outward directed social interaction, this in order to determine deficits in social behaviour, and was performed as previously described (Möller *et al.*, 2011). In short, a pair of unfamiliar rats of the same exposure group and that had the same prior experiences were placed in the centre of an open field arena and allowed to adapt to their surroundings for 30 seconds. Thereafter, time spent participating in several social behavioural categories was recorded for 10 minutes with a digital video camera and scored by Noldus EthoVision© 225 XT software (Noldus Information Technology, Wageningen, Netherlands), as well as a researcher blind to the exposure groups. These behaviours included (1) time spent together, (2) exploration from a distance (approaching the conspecific), (3) social investigation (anogenital sniffing of the conspecific), (4) non-social exploration (rear and supported rear) (see (Blanco-Gandía *et al.*, 2015) for review) and (5) self-directed behaviour (self-grooming and distance moved) (Gonzalez *et al.*, 1996). Each arena was cleaned with a solution containing 10% ethanol after each test.

3.2.6.2 Elevated plus maze (EPM)

The EPM is an extensively validated behavioural assay to determine aversive and/or anxiety behaviour in rodents (Pellow *et al.*, 1985; Walf & Frye, 2007), and is also utilized to determine whether drugs of abuse evoke anxiety responses in rodents (Hogg, 1996). The plus-shaped maze has two alternating open arms and two closed arms (each length × width: 50 cm × 10 cm) which are connected by a (10 cm × 10 cm) neutral zone (Wegener *et al.*, 2012). The entire maze is constructed of black plexiglas and elevated 80 cm above the ground. The light intensities of the open and closed arms are 80 lux and 20 lux, respectively (Fischer *et al.*, 2012). Each male SD rat was placed individually on the neutral centre zone with its head facing an open arm (Harvey *et al.*, 2006). To ensure consistency throughout the EPM paradigm, each rat was handled in a consistent manner and placed in the same position (thus facing the same open arm) in the neutral zone (Walf & Frye, 2007). The rats were allowed to freely explore all aspects of the maze for a testing period of 5 minutes. Their behaviour and movement on the maze were recorded using a digital video camera mounted on the ceiling above the maze and connected to a computer. EthoVision© 225 XT software (Noldus Information Technology, Wageningen, Netherlands) was used to score the following behavioural parameters: (1) % time spent on the open arms, (2) the number of entries onto the open arms, indicative of the rat's inherent nature to explore novel environments (anxiolytic behaviour); (3) % time spent on the closed arms and (4) the number of entries onto the closed arms, indicative of the rat's proclivity towards the dark, enclosed arms of the maze (Montgomery, 1955) (anxiogenic behaviour). Following each test session, the mazes were cleaned with a 10% ethanol solution.

3.2.6.3 Pre-pulse inhibition (PPI) test

PPI of the acoustic startle reflex is used as an operational measure of possible deficits in sensorimotor gating, a well-described behavioural modality with which to study psychosis-related behaviours in rodents and humans (Braff & Geyer, 1990) and response to antipsychotic drugs. However, PPI is also used to assess these symptoms following exposure to drugs of abuse (Boucher *et al.*, 2007; Arai *et al.*, 2008; Todd *et al.*, 2017). The PPI test was performed as described previously (Möller *et al.*, 2013a). Briefly, each SD rat was placed in a ventilated, standard sound-attenuated startle chamber (SRLAB, San Diego Instruments, San Diego, USA) and habituated with 68 dB background white noise (which continued throughout the entire PPI test session) for a period of 5 minutes. A total of 70 PPI test trials were performed with an average interval of 25 seconds. These PPI trials included a 20 ms non-startling prepulse (with intensities of 72 dB, 76 dB, 80 dB or 84 dB) followed by a single 40 ms startle stimulus (with an intensity of 115 dB) 80 ms later. PPI was performed in 4 BLOCKS: the first 10 pulse-alone stimuli (BLOCK 1); 20 pulse-alone stimuli (BLOCK 2 and BLOCK 3); the last 10 pulse-alone stimuli (BLOCK 4), in order to obtain a measure of mean startle amplitude, which shows possible habituation to the continuous delivery of startling stimuli (Van den Buuse & Eikelis, 2001). The % PPI for all the

prepulse intensities were then calculated using the following formula: $\%PPI = \frac{\text{Startle response for Pre-pulse + Pulse trail}}{\text{Startle response for Pulse trail alone}} \text{ X } 100$ (Van den Buuse & Eikelis, 2001).

3.2.7 Peripheral immune-neurochemistry analysis

3.2.7.1 Blood collection

After humane euthanization (via decapitation), trunk blood was collected in pre-chilled Vacuette® tubes containing a K_2EDTA solution as anti-coagulant and centrifuged (14 000 rotations per minute for 10 minutes at 4°C) (Möller *et al.*, 2013a). The plasma was stored at -80°C (Laboratory for Applied Molecular Biology (LAMB), NWU) until the day of analysis. When conducting the analysis, the plasma samples were thawed on ice and centrifuged again (as described above) (Möller *et al.*, 2013a).

Analysis of plasma cytokines

Plasma anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine TNF-α levels were measured in duplicate using sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacture's instructions (Biocom Biotech, Centurion, South Africa). Briefly, 100 μl (for IL-10 analysis) or 50 μl (for TNF-α analysis) of plasma sample were added to each well of a 96 - monoclonal anti-body coated - well plate. After removal of the liquid, a Biotinylated Detection Antibody was added to the wells. Thereafter the wells were washed and an Avidin-Horseradish Peroxidase (HRP) conjugate, which binds to the Biotinylated Antibody, was added to complete the 4-layer sandwich. The wells were analysed using a microplate reader with a 450 nm wavelength filter (Möller *et al.*, 2013a). The cytokine concentrations in the samples were calculated using a standard curve obtained from the known reference standard supplied by the manufacturer (Möller *et al.*, 2013a).

Analysis of tryptophan metabolites

Plasma tryptophan metabolism was determined as described previously (Badawy & Morgan, 2010; Möller *et al.*, 2012b). Each solid phase extraction cartridge (Waters Oasis HLB Extraction Cartridges, Microsep Pty Ltd., Sandton, South Africa) was conditioned with 2 ml methanol (MeOH) and 2 ml 1% formic acid (FA). Sample preparation entailed the addition of 100 μl of the intern standard (anthranilic acid isopropylamide), 400 μl distilled water and 100 μl perchloric acid to 800 μl plasma sample, with these reaction vessels kept on ice. The samples were vortexed for 10 seconds and allowed to sit for 5 minutes. Thereafter the samples were centrifuged (14 000 rpm for 10 minutes at 4°C) and the supernatant loaded onto the extraction column. Each extraction

cartridge was then washed with 2 ml 1% FA and vacuum-dried for 5-10 minutes at ±20 kPa. The contents were then eluted with 3.5 ml 1% NH_4OH in MeOH: tetrahydrofuran evaporated under a gentle stream of nitrogen and reconstituted with 125 μl 25% MeOH: distilled H_2O. The analytes were detected and monitored at a wavelength of 220 nm by injecting 100 μl into a high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA, USA). The concentrations of kynurenine, tryptophan, QA and KYNA were determined using a concentration range between 10 ng/ml-200 ng/ml. The KYNA and kynurenine concentrations were used to calculate the neuroprotective ratio using the formula: [1000 × plasma KYNA (mM)] / plasma kynurenine (mM) (Myint *et al.*, 2007; Myint *et al.*, 2011; Möller *et al.*, 2012a).

3.2.7.2 Brain dissection

Stereological co-ordinates were used to identify the hippocampus as previously described (Paxinos & Watson, 1998). Thereafter, the hippocampus was carefully dissected from the fresh brain, snap frozen in liquid nitrogen and stored at -80°C (LAMB, NWU) until the day of hippocampal OT analysis.

Hippocampal OT

Quantification of hippocampal OT levels was performed in duplicate using enzyme-linked immunosorbent assay (ELISA) kits (Elabscience®, Biocom Biotech, Centurion, South Africa). Briefly, the hippocampal tissue was thawed on ice and homogenized in ice-cold phosphate-buffered saline (PBS). Thereafter, 50 µl of the homogenate and 50 µl biotinylated detection antibody were added to each well of a 96-monoclonal anti-body coated- well plate. The wells were washed and an HRP conjugate was added to each well. The optical density of each well was then determined using a microplate reader with a 450 nm wavelength filter. The OT levels in the samples were then calculated using a standard curve obtained from the known reference standard supplied by the manufacturer.

3.2.8 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 was done under the guidance of the Statistical Consultation Service of the NWU. Histograms, Q-Q plots and the Shapiro Wilk test were used to test for normality. In order to compare the EFV, Δ^9-THC and EFV + Δ^9-THC exposure groups with one another, one-way analysis of variance (ANOVA) was carried out with appropriate post hoc testing performed using Bonferroni only if F achieved statistical significance (Möller *et al.*, 2013b). However, animal body weight (mean ± standard error of the mean (SEM)) was analysed by two-way ANOVA (with drug

exposure and days of weight as the two factors) with repeated measures for different days of weight measures followed by Bonferroni post hoc analyses. % PPI was also analysed by two-way ANOVA (with drug exposure and decibel (dB) intensity as the two factors) followed by Bonferroni post hoc test with repeated measures for the different pre-pulse intensities (Möller *et al.*, 2013a). In all cases data are expressed as the mean ± SEM, where a p-value of <0.05 is considered as statistically significant.

3.3 Results

3.3.1 Body weight

Significant and equal growth were observed in all exposure groups over the sub-chronic study period with no significant group differences observed with regards to drug- (EFV, Δ^9-THC and EFV + Δ^9-THC) or vehicle (pharmaceutical grade olive oil) exposure (data not shown).

3.3.2 Behavioural analysis

3.3.2.1 Social interaction test (SIT)

Total distance moved

One-way ANOVA analysis revealed a significant main effect of drug exposure on the total distance moved ($F(3, 40) = 3$, $p = 0.0209$). Bonferroni post hoc testing with multiple comparisons indicated a significant decrease in the total distance moved in the EFV + Δ^9-THC exposure group when compared to the vehicle control ($p = 0.0017$), the EFV exposure group ($p = 0.0177$) and the Δ^9-THC exposure group ($p < 0.0001$) (Fig. 3-1A). The separate EFV and Δ^9-THC exposure groups did not differ from the control, nor did EFV exposure differ from exposure to Δ^9-THC alone (Fig. 3-1A).

Time spent self-grooming

One-way ANOVA analysis revealed a significant main effect of drug exposure on the time spent self-grooming ($F(3, 76) = 4$, $p = 0.0051$). Bonferroni post hoc testing with multiple comparisons indicated a significant increase in the time spent self-grooming in the EFV - ($p = 0.047$) and Δ^9-THC exposure groups ($p = 0.0191$) when compared to the vehicle control (Fig. 3-1B). The EFV + Δ^9-THC exposure group did not differ from control (Fig. 3-1B). EFV and Δ^9-THC exposure alone did not differ from the combination of EFV +Δ^9-THC, nor did exposure to EFV differ from Δ^9-THC exposure (Fig. 3-1B).

Time spent rearing

One-way ANOVA analysis revealed a significant main effect of drug exposure on the time spent rearing ($F(3, 76) = 8$, $p < 0.0001$). Bonferroni post hoc testing with multiple comparisons indicated a significant increase in the time spent rearing in the EFV exposure group ($p = 0.0071$) and in the Δ^9-THC exposure group ($p = 0.0059$) when compared to the vehicle control, although the EFV + Δ^9-THC exposure group did not differ from control (Fig. 3-1C). The EFV + Δ^9-THC exposure group differed significantly vs. either group alone ($p = 0.0029$ and $p = 0.0023$, respectively), whilst EFV exposure did not differ from Δ^9-THC exposure alone (Fig. 3-1C).

Total time spent together

One-way ANOVA analysis revealed a significant main effect of drug exposure on the total time spent together ($F(3, 40) = 3$, $p = 0.0209$). Bonferroni post hoc testing with multiple comparisons indicated a significant increase in the total time spent together in the EFV + Δ^9-THC exposure group ($p = 0.047$) when compared to the vehicle control, with the EFV + Δ^9-THC exposure group significantly higher vs. the EFV exposure group ($p = 0.0333$) but not the Δ^9-THC exposure group (Fig. 3-1D). The separate EFV and Δ^9-THC exposure groups did not differ from the control, nor did EFV exposure differ from Δ^9-THC exposure (Fig. 3-1D).

Times approaching each other

One-way ANOVA analysis revealed a significant main effect of drug exposure on times approaching each other ($F(3, 40) = 5$, $p = 0.0028$). Bonferroni post hoc testing with multiple comparisons indicated a significant increase in times approaching each other in the EFV + Δ^9-THC exposure group ($p = 0.0301$) when compared to the vehicle control ($p = 0.0301$) and the EFV exposure group ($p = 0.003$) but not vs. Δ^9-THC exposure group (Fig. 3-1E). Separate exposure to EFV and Δ^9-THC did not differ from the vehicle, nor did EFV exposure differ from Δ^9-THC exposure (Fig. 3-1E).

Time spent anogenital sniffing

One-way ANOVA analysis revealed a significant main effect of drug exposure on time spent anogenital sniffing ($F(3, 76) = 6$, $p = 0.0009$). Bonferroni post hoc testing with multiple comparisons indicated a significant decrease in the EFV exposure group ($p = 0.002$) when compared to the vehicle control ($p = 0.002$), the Δ^9-THC exposure group ($p = 0.0061$) and the EFV + Δ^9-THC exposure group ($p = 0.0063$) (Fig. 3-1F). Exposure to Δ^9-THC alone and EFV + Δ^9-THC did not differ from the vehicle, nor did Δ^9-THC exposure differ from EFV+ Δ^9-THC exposure (Fig. 3-1F).

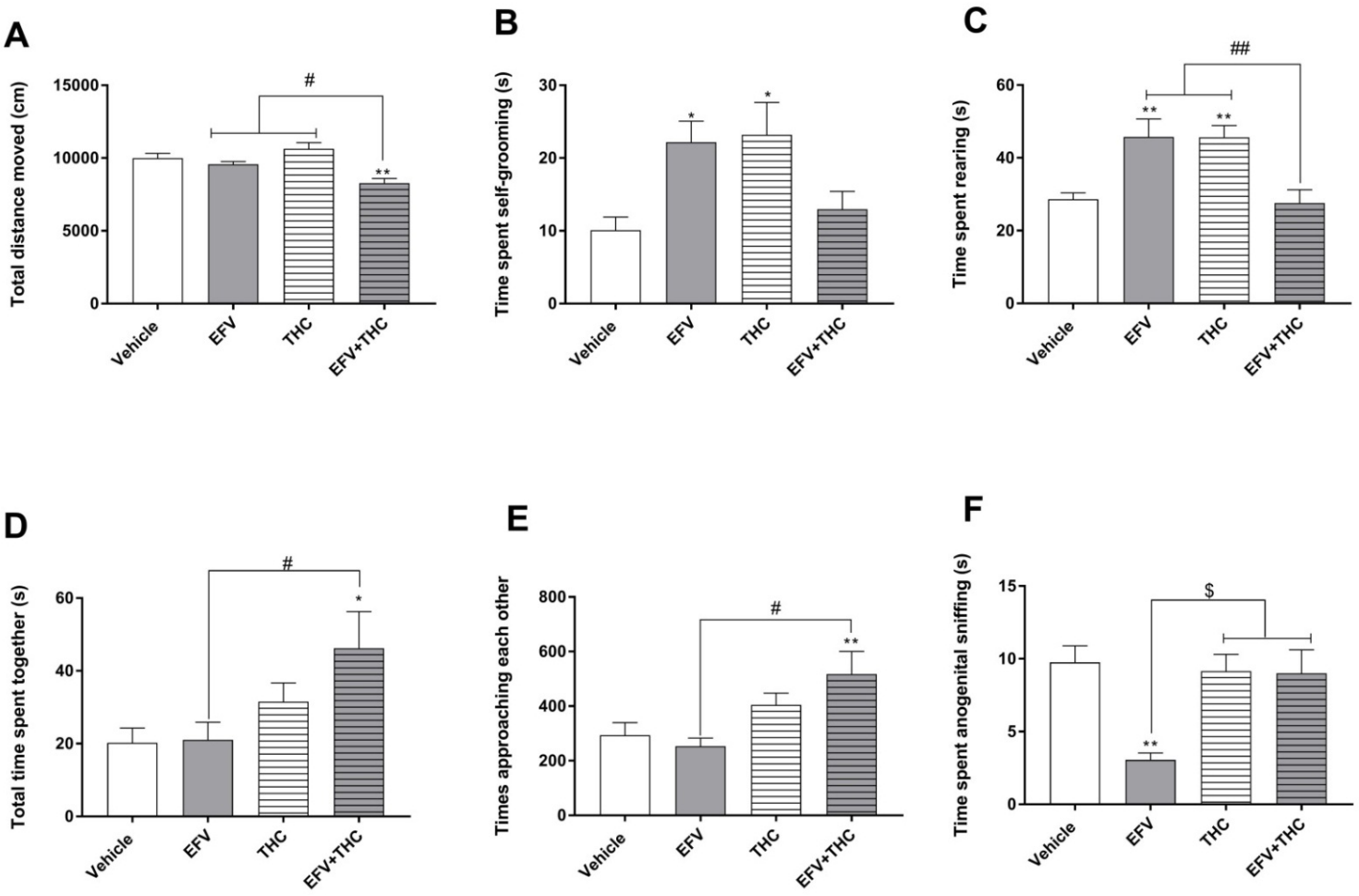

Fig. 3-1: Self-directed (A-C) and social interactive (D-F) behaviour in the respective exposure groups with (A) Total distance moved; (B) Time spent self-grooming; (C) Time spent rearing; (D) Total time spent together; (E) Times approaching each other; (F) Time spent anogenital sniffing. *p < 0.05, **p < 0.01 vs. Vehicle; #p < 0.05, ##p < 0.01 vs. EFV or EFV + Δ^9-THC; $p < 0.01 vs. Δ^9-THC and EFV + Δ^9-THC (Bonferroni post hoc test). Refer to text for precise *p* values.

3.3.2.2 Elevated plus maze test (EPM)

Entries into open arms

One-way ANOVA analysis revealed a significant main effect of drug exposure on entries into the open arms ($F(3, 66) = 7$, $p = 0.0002$). Bonferroni post hoc testing indicated a significant increase in entries into the open arms in the Δ^9-THC exposure group when compared to the vehicle control ($p = 0.0002$), although the other exposure groups did not differ vs. control (Fig. 3-2A). The Δ^9-THC exposure group was also significantly higher vs. the EFV exposure group ($p = 0.027$) and to the EFV + Δ^9-THC exposure group ($p = 0.002$) (Fig. 3-2A). The EFV exposure group did not differ from the EFV + Δ^9-THC exposure group (Fig. 3-2A).

Entries into closed arms

One-way ANOVA analysis revealed a significant main effect of drug exposure on entries into closed arms ($F(3, 66) = 2.25$, $p = 0.045$). Bonferroni post hoc testing indicated a significant decrease in entries into the closed arms in the Δ^9-THC exposure group when compared to the vehicle control ($p = 0.041$), although neither of the other exposure groups differed in this regard (Fig. 3-2B). The Δ^9-THC exposure group was significantly lower vs. the EFV +Δ^9-THC group ($p = 0.034$) (Fig 3-2B).

% Time in open arms

One-way ANOVA analysis revealed a significant main effect of drug exposure on % time in the open arms ($F(3, 66) = 2$, $p = 0.0446$). Bonferroni post hoc testing with multiple comparisons indicated a significant decrease in % time in the open arms in the EFV exposure group when compared to the vehicle control ($p = 0.0311$), although neither of the other exposure groups differed in this regard (Fig. 3-2C). The EFV exposure group was significantly lower vs. the Δ^9-THC exposure group ($p = 0.04$) (Fig. 3-2C). Separate exposure to EFV and Δ^9-THC did not differ from exposure to EFV + Δ^9-THC (Fig. 3-2C).

% Time in closed arms

One-way ANOVA analysis revealed a significant main effect of drug exposure on % time in the closed arms ($F(3, 66) = 3$, $p = 0.0146$). Bonferroni post hoc testing with multiple comparisons indicated a significant increase in % time in the closed arms in the EFV exposure group ($p = 0.039$) and a significant decrease in the Δ^9-THC exposure group ($p = 0.02$) when compared to the vehicle control (Fig. 3-2D). The EFV + Δ^9-THC exposure group did not differ vs. control or either of the other exposure groups (Fig. 3-2D). The EFV exposure group was significantly elevated vs. the Δ^9-THC exposure group ($p = 0.0084$) (Fig. 3-2D).

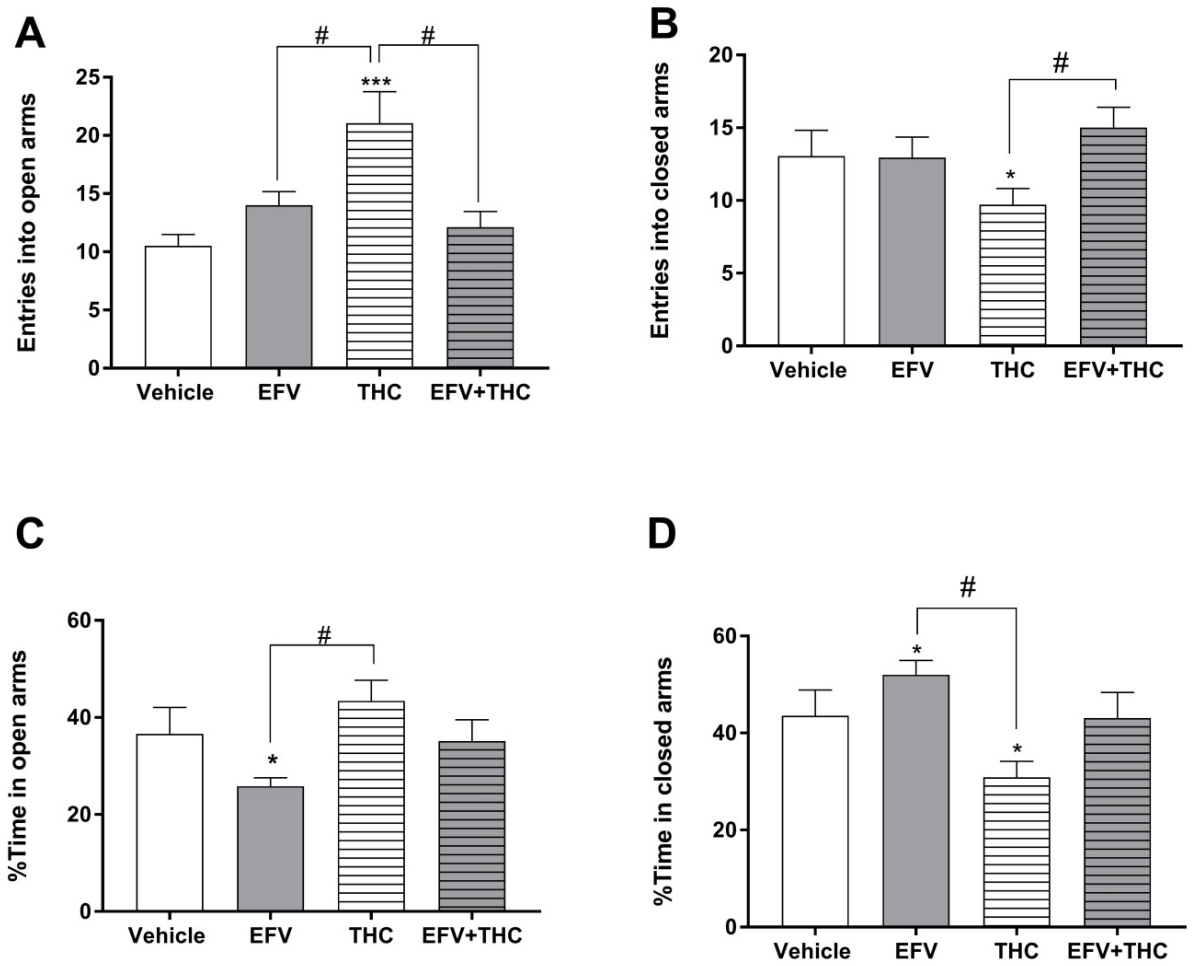

Fig. 3-2: Elevated plus maze in rats exposed to the respective drugs as indicated with (A) Entries into open arms; (B) Entries into closed arms; (C) %Time in open arms and (D) % Time in closed arms. *p < 0.05, ***p < 0.001 vs. Vehicle; #p < 0.05 vs. Δ^9-THC (Bonferroni post hoc test). Refer to text for precise *p* values.

3.3.2.3 Pre-pulse inhibition

Two-way ANOVA analysis revealed a significant interaction between drug exposure and dB intensity ($F(9, 195) = 5.024$, $p < 0.0001$) and a significant main effect of drug exposure ($F(3, 65) = 15.79$, $p < 0.0001$) and dB intensity ($F(3, 195) = 303$, $p < 0.0001$) on % PPI. Bonferroni post hoc testing with multiple comparisons indicated a significant decrease in % PPI in the EFV exposure group at 72 dB ($p = 0.0007$) and 76 dB ($p = 0.0008$) compared to the vehicle control, and at 72 dB ($p < 0.0001$) when compared to the EFV + Δ^9-THC exposure group (Fig. 3-3). The EFV and Δ^9-THC exposure groups did not differ vs. one another at any of the dB intensities (Fig. 3-3). Bonferroni post hoc testing with multiple comparisons also indicated a significant decrease in % PPI in the Δ^9-THC exposure group when compared to the vehicle control at 84 dB ($p = 0.0310$), and at 72 dB ($p < 0.0001$) and 76 dB ($p = 0.0146$) when compared to the EFV + Δ^9-THC exposure group (Fig 3-3). Bonferroni post hoc analysis with multiple comparisons also indicated

a significant decrease in % PPI in the EFV + Δ^9-THC exposure group at 72 dB ($p < 0.0001$), 76 dB ($p < 0.0001$), 80 dB ($p = 0.0090$) and 84 dB ($p < 0.0001$) compared to the vehicle control, although it did not show differences when compared to the EFV and Δ^9-THC exposure groups at 76 dB, 80 dB and 84 dB (Fig. 3-3). Neither exposure to EFV nor Δ^9-THC alone showed differences at 80 dB and 84 dB (for EFV) as well as 76 dB and 80 dB (for Δ^9-THC) when compared to the vehicle control (Fig. 3-3).

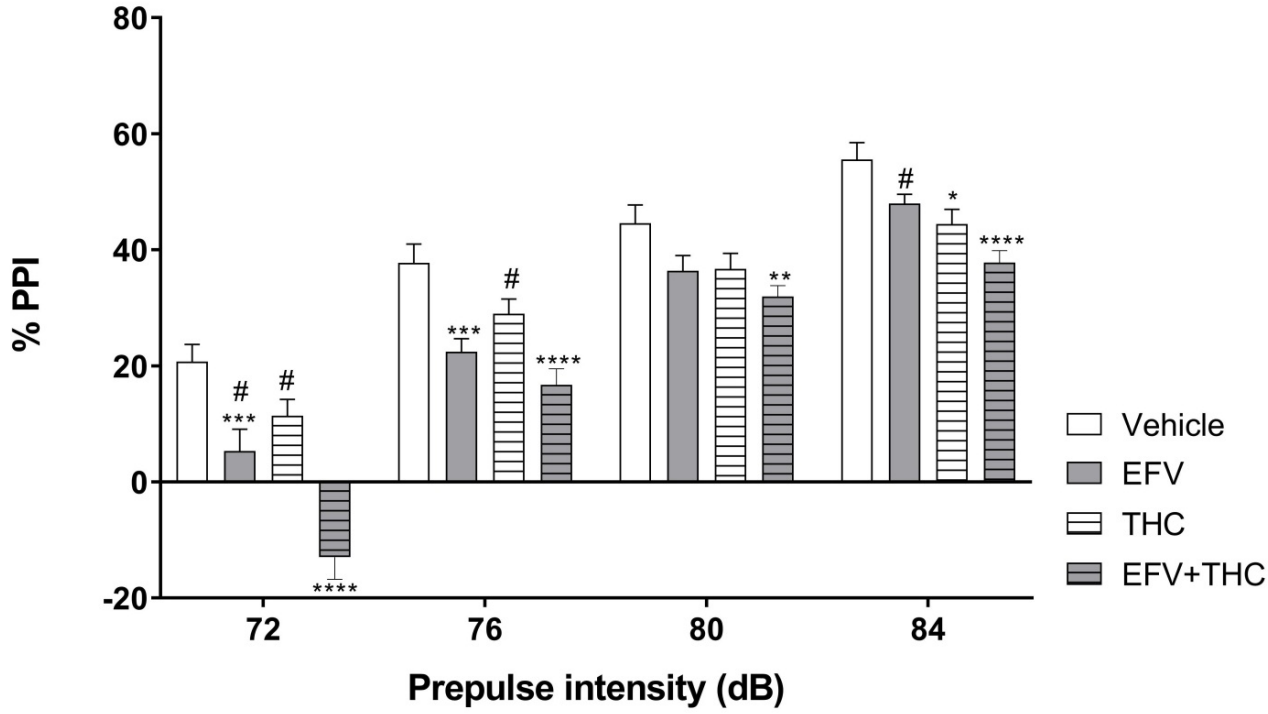

Fig. 3-3: Percentage prepulse inhibition (PPI) in rats exposed to the specific drugs as indicated at 72, 76, 80 and 84 dB respectively. **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. Vehicle; #p < 0.05 vs. EFV + Δ^9-THC (Bonferroni post hoc test). Refer to text for precise *p* values.

3.3.3 Peripheral plasma analysis

3.3.3.1 Cytokines

*Tumor necrosis factor alpha (*TNF-α*) concentration*

One-way ANOVA analysis revealed a significant main effect of drug exposure on the TNF-α concentration ($F (3, 66) = 4$, $p = 0.0082$). Bonferroni post hoc testing with multiple comparisons indicated a significant increase in TNF-α concentration in the EFV exposure group when compared to the vehicle control ($p = 0.0106$), although none of the other exposure groups evoked a noteworthy change in this pro-inflammatory cytokine (Fig. 3-4A) in comparison to the control. The EFV-induced increase in TNF-α was also significantly higher than of the EFV + Δ^9-THC

exposure group (p = 0.0316) (Fig. 3-4A). No differences were observed between exposure to Δ^9-THC vs. EFV and vs. EFV + Δ^9-THC (Fig. 3-4A).

Interleukin-10 (IL-10) concentration

One-way ANOVA analysis revealed a significant main effect of drug exposure on the IL-10 concentration (F (3, 66) = 3, p = 0.0152). Bonferroni post hoc testing with multiple comparisons indicated a significant decrease in the IL-10 concentration in the Δ^9-THC exposure group (p = 0.0304) when compared to the vehicle control (p = 0.0304), as well as vs. the EFV exposure group (p = 0.029) (Fig. 3-4B). None of the other exposure groups had a marked effect or differences on IL-10 (Fig. 3-4B).

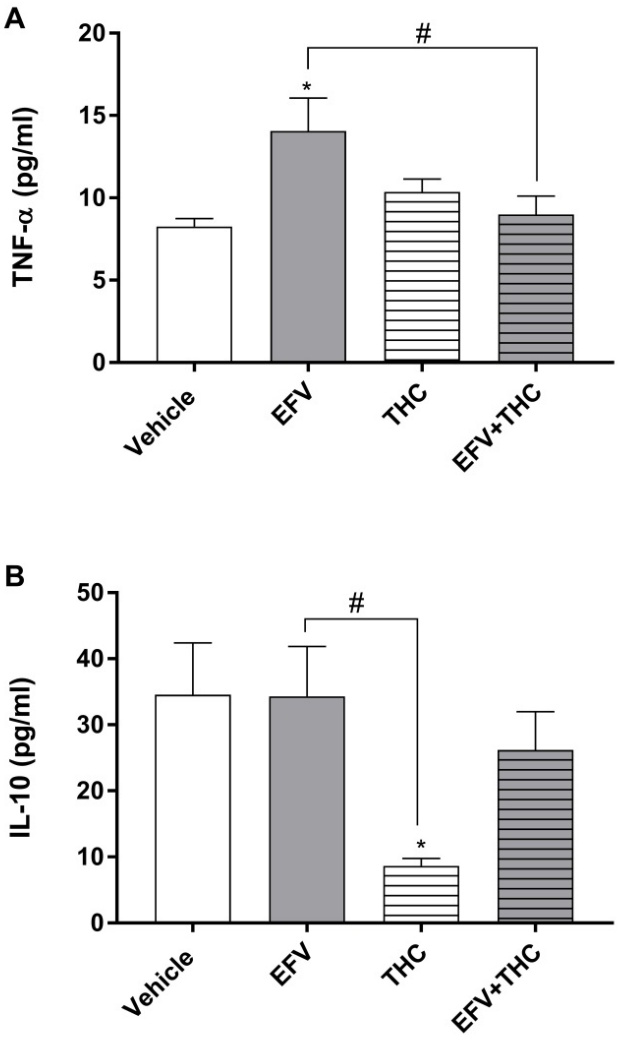

Fig. 3-4: Pro-inflammatory cytokine, TNF-α (A) and anti-inflammatory cytokine, IL-10 (B) plasma concentrations in the respective exposure groups. *p < 0.05 vs. Vehicle; #p < 0.05 vs. EFV (Bonferroni post hoc test). Refer to text for precise *p* values.

3.3.3.2 Kynurenine pathway metabolites and the neuroprotective ratio

Tryptophan

One-way ANOVA analysis revealed a significant main effect of drug exposure on the tryptophan concentration ($F(3, 56) = 6$, $p = 0.0010$). Bonferroni post hoc testing with multiple comparisons indicated a significant decrease in tryptophan in the Δ^9-THC exposure group ($p = 0.0116$) when compared to the vehicle control ($p = 0.0116$), with none of the other treatment groups having a noteworthy effect (Fig. 3-5A). The reduction in tryptophan in the Δ^9-THC exposure group was also significantly reduced vs. the EFV + Δ^9-THC exposure group ($p = 0.001$) (Fig. 3-5A). EFV exposure did not cause any significant differences when compare to Δ^9-THC as well as EFV +Δ^9-THC (Fig. 3-5A).

Kynurenine

No significant effect of drug exposure on the kynurenine concentration was observed (Fig 3-5B).

Kynurenic acid (KYNA

One-way ANOVA analysis revealed a significant main effect of drug exposure on the KYNA concentration ($F(3, 52) = 7$, $p < 0.0001$). Bonferroni post hoc testing with multiple comparisons indicated a significant decrease in KYNA in the EFV exposure group ($p < 0.0001$), the Δ^9-THC exposure group ($p < 0.0001$) and the EFV + Δ^9-THC exposure group ($p < 0.0001$) when compared to the vehicle control, with no significant differences noted between the various drug exposure groups (Fig. 3-5C).

Quinolinic acid (QA)

One-way ANOVA analysis revealed a significant main effect of drug exposure on the QA concentration ($F(3, 57) = 4$, $p = 0.0112$). Bonferroni post hoc testing with multiple comparisons indicated a significant increase in QA in the EFV ($p = 0.042$), the Δ^9-THC ($p = 0.0263$) and the EFV + Δ^9-THC exposure groups ($p = 0.044$) when compared to the vehicle control, with no significant differences noted between the various drug exposure groups (Fig. 3-5D).

Neuroprotective ratio

One-way ANOVA analysis revealed a significant main effect of drug exposure on the neuroprotective ratio ($F(3, 48) = 1$, $p < 0.0001$). Bonferroni post hoc testing with multiple comparisons indicated a significant decrease in the neuroprotective ratio in the EFV exposure

group ($p < 0.0001$), the Δ^9-THC exposure group ($p < 0.0001$) and the EFV + Δ^9-THC exposure group ($p < 0.0001$) when compared to the vehicle control, with no significant differences noted between the various drug exposure groups (Fig. 3-5E).

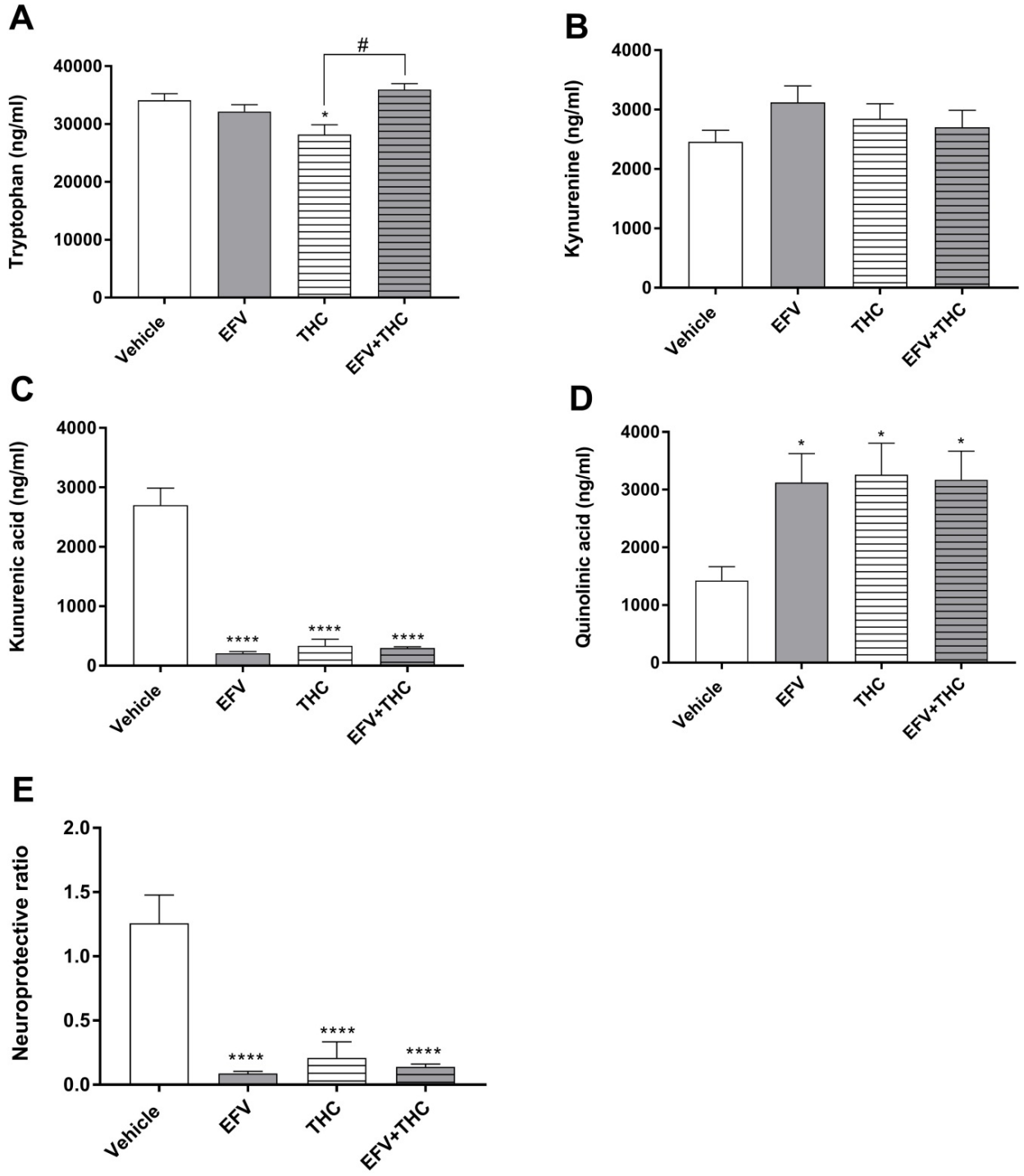

Fig. 3-5: Kynurenine pathway metabolites in the respective exposure groups, with (A) Tryptophan, (B) Kynurenine, (C) Kynurenic acid, (D) Quinolinic acid and (E) Neuroprotective ratio (Kynurenic acid / Kynurenine). *$p < 0.05$, ****$p < 0.0001$ vs. Vehicle; #$p < 0.01$ vs. EFV + Δ^9-THC (Bonferroni post hoc test). Refer to text for precise *p* values.

3.3.4 Neurochemical analysis

3.3.4.1 Hippocampal oxytocin levels

One-way ANOVA analysis revealed a significant main effect of drug exposure on the hippocampal OT levels ($F(3, 66) = 8$, $p < 0.0001$). Bonferroni post hoc testing with multiple comparisons indicated a significant increase in hippocampal OT levels in the EFV + Δ^9-THC exposure group when compared to the vehicle control ($p < 0.0001$), with neither of the drug exposures alone having a noteworthy effect on OT levels (Fig. 3-6). Hippocampal OT levels was also significantly higher in the combined EFV + Δ^9-THC exposure group vs. that of the EFV exposure group ($p = 0.0165$) and vs. the Δ^9-THC exposure group ($p = 0.0017$) (Fig. 3-6). No difference in hippocampal OT levels was observed in the EFV vs. Δ^9-THC exposure groups (Fig. 3-6).

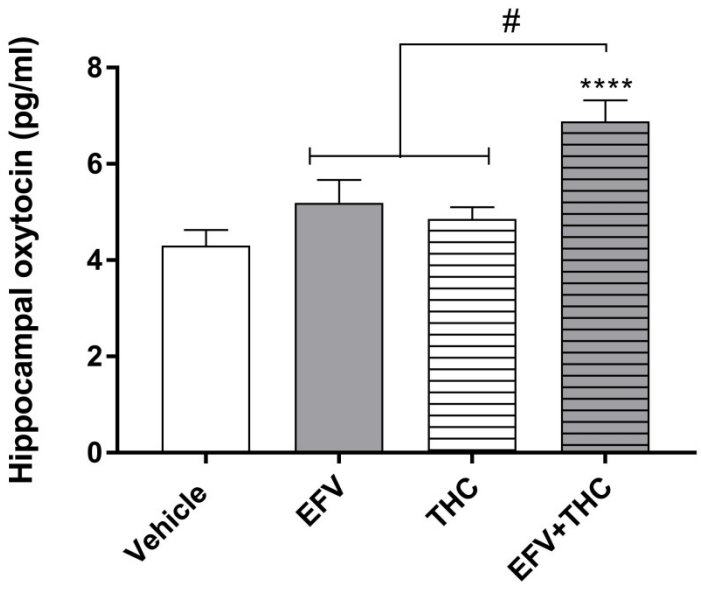

Fig. 3-6: Hippocampal OT levels in the respective exposure groups. ***p < 0.001 vs. Vehicle; #p < 0.05 vs. EFV + Δ^9-THC (Bonferroni post hoc test). Refer to text for precise *p* values.

3.4 Discussion

The major findings in this study are that sub-chronic exposure to EFV (5 mg/kg) induces significant deficits in selected social interactive behaviours, and induces anxiogenic and psychotogenic-like behaviour. Sub-chronic exposure to Δ^9-THC (0.75 mg/kg) also induces selected deficits in social behaviour, induces psychotogenic-like behaviour but *decreases* anxiety-like behaviour. Combined EFV + Δ^9-THC exposure increased pro-social and psychosis-related behaviour. Exposure to both EFV and Δ^9-THC alone induced a pro-inflammatory state by

opposing actions on pro- and anti-inflammatory cytokines, viz. increasing plasma TNF-α (EFV) and decreasing plasma IL-10 (Δ^9-THC) levels, respectively. Δ^9-THC increased tryptophan conversion (lowered plasma tryptophan) in favour of QA synthesis evinced as increased QA and decreased KYNA, the latter also evident in the EFV and combined EFV + Δ^9-THC group. Importantly, all three exposure groups reduced the neuroprotective ratio. We also found that the combination of EFV + Δ^9-THC increased hippocampal OT levels.

The social interaction data indicates that sub-chronic exposure to both EFV and Δ^9-THC induces social withdrawal with increased anti-social exploration (rearing) and self-directed behaviours (time spent self-grooming) as well as decreased anogenital sniffing in the EFV exposure group. Self-grooming (which can be regarded as non-social self-exploratory behaviour) potentially serves as an adaptive response and coping mechanism to initiate stress-reduction during the social interaction test (Blanchard *et al.*, 2001; Kalueff & Tuohimaa, 2005; Schneider *et al.*, 2008). The significant increase in time spent rearing is indicative of a profound increase in non-social investigatory behaviour, further promoting the anti-social behavioural profile of both EFV and Δ^9-THC. These findings are in accordance with previous preclinical studies reporting that chronic exposure to cannabinoid receptor agonists (such as WIN 55, 212-2, cannabidiol and Δ^9-THC) also promote social withdrawal and/or decrease social interaction by increasing anti-social behavioural parameters (Ando *et al.*, 2006; O'Tuathaigh *et al.*, 2008; O'Tuathaigh *et al.*, 2010; Almeida *et al.*, 2013; Katsidoni *et al.*, 2013). In fact one study specifically reported that chronic cannabis administration can induce long-lasting impairments in social behaviour (Gruber & Pope Jr, 2002). However, Δ^9-THC exposure had no significant effect on the time spent anogenital sniffing (which can be regarded as a pro-social behavioural parameter (Blanco-Gandía *et al.*, 2015)), whilst EFV exposure significantly decreased this parameter, suggesting a more pronounced anti-social behavioural profile for EFV. Interestingly, the combination of these drugs (EFV + Δ^9-THC) promoted social interaction as indicated by a significant increase in the time that the unfamiliar rat pair spent together as well as the times they were approaching each other. The combination of EFV + Δ^9-THC also caused a significant decrease in locomotor activity by reducing the total distance moved during the social interaction test. The observed hypoactivity could be attributed to a decrease in exploratory behaviour, possibly due to the fact that the rats spent more time engaging in social interactive behaviour as well as social exploration.

The exact mechanism/s whereby EFV, Δ^9-THC and the combination of EFV + Δ^9-THC mediate different aspects of social behaviour are not known, but emphasizes just how drugs of abuse may *differently* affect this important aspect of human behaviour. Given how the pharmacological profile of these drugs differ may understandably underlie these differences. Consequently we have considered the involvement of the peptide hormone and neuromodulator, OT, in modulating these behaviours. The central release of OT has been implicated in both pro-social behaviour

and anxiolytic responses (McRae-Clark *et al.*, 2013). A significant increase in hippocampal OT levels was only observed in the EFV + Δ^9-THC exposure group, while this increase would account for increased pro-social behaviour noted with this combination, viz. times approaching and total time spent together. Several clinical and preclinical studies have ascribed the pro-social effects of illicit drugs such as psychostimulants and 3,4-methylenedioxymethamphetamine (MDMA) to OT (Thompson *et al.*, 2007; Dumont *et al.*, 2009; Sarnyai, 2011). This is a particular relevant finding, suggesting a pro-social behavioural profile for "Nyaope"/"Whoonga" (known to contain both EFV and Δ^9-THC (Marwaha, 2008; Hull, 2010; Cullinan, 2011; Fihlani, 2011)).

It has been widely accepted that an increase in social interaction (without a concomitant increase in locomotor activity) is indicative of anxiolytic effects, with the opposite indicative of an anxiogenic effect (File & Hyde, 1978; File & Seth, 2003; Blanco-Gandía *et al.*, 2015). Sub-chronic exposure to EFV induced significant anxiogenic behaviour by decreasing the % time spent in the open arms and increasing the % time spent in the closed arms of the EPM. This is in line with previous preclinical studies reporting anxiogenic effects for chronic EFV exposure, albeit at different doses, viz. 50 mg/kg (Cavalcante *et al.*, 2017) and 10 mg/kg (Romao *et al.*, 2011). Considering the fact that Δ^9-THC prompted an increase in self-directed social interaction, i.e. anti-social behaviour, one would expect it to exert an anxiogenic effect in the EPM test. However, sub-chronic exposure to Δ^9-THC promoted anxiolytic behaviour by increasing the entries into the open arms and decreasing both the entries into and % time in closed arms. The conflicting SIT and EPM results could be attributed to the Δ^9-THC dose (0.75 mg/kg) used in our experiments, with one study specifically reporting a dose-dependent anxiolytic effect of Δ^9-THC between 0.075 mg/kg and 1.5 mg/kg, with 0.75 mg/kg being the most effective in initiating anxiolytic effects (Rubino *et al.*, 2015). Another preclinical study also reported a dose-dependent biphasic effect of Δ^9-THC on anxiety response with exposure to high doses (5 mg/kg) promoting anxiogenic behaviour whilst exposure to lower doses (0.3 mg/kg) promotes anxiolytic behaviour (Valjent *et al.*, 2002). The anxiolytic effects of Δ^9-THC in the EPM could also be attributed to its ability to activate the endogenous cannabinoid system by activating cannabinoid CB_1 receptors, which is known to ameliorate fear responses under highly aversive situations through the modulation of glutamate release (Monory *et al.*, 2006; Kamprath *et al.*, 2009; Moreira & Wotjak, 2009).

More on our anxiety findings, several preclinical studies have attributed anxiety-like behaviour to alterations in regional brain monoamine levels, specifically increased DA (in the medial prefrontal cortex and striatum) (Cenci *et al.*, 1992; Tidey & Miczek, 1996; Lyss *et al.*, 1999; Cavalcante *et al.*, 2017) and NE (in the ventral hippocampus and striatum) (Joca *et al.*, 2007; Cavalcante *et al.*, 2017) as well as elevated 5-HT levels (in the ventral hippocampus and striatum) (Watt *et al.*, 2009; Cavalcante *et al.*, 2017). Interestingly, we recently established that sub-chronic exposure to EFV significantly increases DA levels in the prefrontal cortex of rats and increases both striatal 5-HT

and NE levels (Möller *et al.*, 2018). We posit that these monoaminergic effects of EFV contribute to the above-noted anxiogenic profile. However, Möller *et al.* (2018) also reported reduced levels of NE and 5-HT in the prefrontal cortex as well as decreased hippocampal 5-HT following sub-chronic Δ^9-THC exposure, possibly explaining the observed anxiolytic profile of this drug. The combination of EFV + Δ^9-THC did not produce any significant effect in the EPM test, which could be explained by opposing actions of EFV and Δ^9-THC on various pathways mediating anxiety.

Drugs of abuse such as methamphetamine (MA) and lysergic acid diethylamide (LSD) are known to cause significant disruptions in PPI resulting in deficits in sensorimotor gating (Ouagazzal *et al.*, 2001; Arai *et al.*, 2008; Strauss *et al.*, 2014; Swanepoel *et al.*, 2017). Sensorimotor gating can be defined as a neurological filtering process by which insignificant or excessive stimuli are either processed or 'filtered' out of consciousness (Braff & Geyer, 1990; Arai *et al.*, 2008). Indeed, a psychological profile has been reported in "Nyaope" addicts (Marwaha, 2008). In our experiments, we observed a significant reduction in % PPI in all the drug exposure groups. EFV exposure significantly decreased % PPI at 72 dB and 76 dB, whilst Δ^9-THC exposure only produced a significant decrease in % PPI at 84 dB. The reduction in % PPI produced by the combination of EFV + Δ^9-THC was even more pronounced since it produced deficits in % PPI at all prepulse intensities studied, while the bolstering effect of the combination was significantly more pronounced vs. EFV (at 84 dB), Δ^9-THC (at 76 dB) and both EFV and THC (at 72 dB). Deficits in sensorimotor gating have been linked to alterations in monoaminergic systems that in turn reflects mechanisms whereby psychosis develop in people abusing these drugs (Braff & Geyer, 1990; Cadenhead, 2011), e.g. mesolimbic hyperdopaminergia (Todd *et al.*, 2017), striatal hypernoradrenergia (Brand *et al.*, 2015). In fact, Möller *et al.* (2018) reported increased DA and NE levels in the striatum of rats exposed to EFV alone and to EFV + Δ^9-THC but not in rats exposed to Δ^9-THC alone, thus reinforcing the above-noted conclusion that more pronounced sensorimotor deficits follows combined use of these drugs.

The above-mentioned neuropsychiatric behavioural profile evoked by EFV abuse could be attributed to EFV-induced neuroinflammation. Indeed, the hydroxylation of EFV by cytochrome 2B6 leads to the formation of 8-hydroxyefavirenz (8-OH-EFV) – a known pro-oxidant and potent neurotoxin that has been especially implicated in EFV-associated neuropsychiatric symptoms (Ogburn *et al.*, 2010; Tovar-y-Romo *et al.*, 2012; Decloedt & Maartens, 2013; Dalwadi *et al.*, 2018). We show here that sub-chronic EFV exposure induces a pro-inflammatory state by significantly increasing plasma TNF-α levels, which promotes neuroinflammation (Clark *et al.*, 2013). These findings are in line with previous preclinical studies also reporting increased plasma TNF-α following EFV exposure (O'Mahony *et al.*, 2005b; Romao *et al.*, 2011). Neuroinflammation produced by pro-inflammatory cytokines (such as TNF-α) is under negative control by anti-inflammatory cytokines (such as IL-10) (Cassatella *et al.*, 1993; Zhang & An, 2007). Importantly,

EFV did not produce a reactive increase in protective plasma IL-10 levels, thereby bolstering the pro-inflammatory potential of this drug. Δ^9-THC exposure, on the other hand, caused a significant reduction in plasma IL-10 levels which may underlie how it may evoke a pro-inflammatory response, and in accordance with a previous preclinical study (Berdyshev *et al.*, 1997). Despite the apparent pro-inflammatory actions for EFV and Δ^9-THC separately, no pro- or anti-inflammatory actions was evident with the combination of EFV + Δ^9-THC. However, it is not unlikely that the combination may induce a pro-inflammatory state by increasing or decreasing other pro- (e.g. Interleukin-2 and Interferon-alpha) (Capuron & Miller, 2011) and anti- (e.g. Interleukin-4) inflammatory cytokines (Zhang & An, 2007) respectively, which warrants future investigation.

We show here for the first time that the pro-inflammatory state induced by sub-chronic EFV, Δ^9-THC and their combination shifts tryptophan metabolism in favour of QA synthesis via the kynurenine pathway. The formation of KYNA and QA from kynurenine in an *appropriate ratio* provides the necessary neuroprotective balance necessary to prevent neurotoxicity that may ensure as a result of neuroinflammation (Möller *et al.*, 2012a). The mechanism whereby EFV and Δ^9-THC increase QA levels and reduce KYNA and the neuroprotective ratio could be attributed to their ability to increase plasma TNF-α and decrease plasma IL-10 levels. Increased TNF-α and decreased IL-10 levels are known to modulate IDO, with pro-and anti-inflammatory cytokines activating and inhibiting this enzyme, respectively (Möller *et al.*, 2015). This action would thereby shift tryptophan metabolism away from serotonin synthesis in favour of kynurenine metabolism. These effects on tryptophan-kynurenine metabolism could also be attributed to increased oxidative stress, as indicated previously (Moller et al., 2018). Most drugs of abuse induce oxidative stress and inflammation which could possibly activate the kynurenine pathway (Cunha-Oliveira *et al.*, 2013). Indeed, Möller *et al.* (2018) reported increased peripheral oxidative stress induced by sub-chronic exposure to EFV (5 mg/kg), Δ^9-THC (0.75 mg/kg) and combined EFV + Δ^9-THC, which could possibly explain the decreased neuroprotective ratio's produced by these drugs. Finally, 5-HT is intimately involved in the genesis of anxiety (Millan, 2003). The resulting diminution of serotonin following the redirecting of tryptophan metabolism via the kynurenine pathway, as demonstrated especially following Δ^9-THC and to some extent EFV, may also underlie the observed anxiety manifestations (Millan, 2003), as noted following EFV exposure.

3.5 Conclusion

EFV-induced deficits in social interaction and sensorimotor-gating as well as anxiety-like behaviour are associated with a pro-inflammatory state, evinced by increased pro-inflammatory cytokine (TNF-α) levels leading to altered tryptophan metabolism, elevated QA and reduced KYNA and a subsequent decrease in the neuroprotective ratio. Moreover, the behavioural profile

of Δ^9-THC is similar to that of EFV alone with the exception of anxiolytic behaviour and decreased IL-10 levels. The combination of EFV + Δ^9-THC specifically increases hippocampal OT levels which may underlie increased social interactive behaviour in this group. Importantly, the combination appears to be superior to EFV or Δ^9-THC alone with regard to inducing psychotogenic-like behaviour. Both the latter findings would explain the psychological profile of "Nyaope". This work proposes possible behavioural, peripheral and neuroendocrine mechanisms underlying the neuropsychiatric manifestations related to the addictive profile and abuse properties of EFV alone or in combination with Δ^9-THC.

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CHAPTER 4

SUMMARY, RECOMMENDATIONS AND CONCLUSION

This chapter will discuss and summarize the results of this study (as presented in Chapter 3). The study aims and hypothesis will also be taken into account in order to provide a detailed account of what has been achieved, make recommendations for future research and hence draw a final conclusion.

4.1 Study aims and relevant outcomes / summary of results

The psychological profile of efavirenz (EFV) and its recreational pattern of abuse has been described (Marwaha, 2008; Sciutto, 2009). EFV tablets are crushed and mixed with common household items and other illicit drugs such as marijuana (with Δ^9-tetrahydrocannabinol (Δ^9-THC) being the psychological component of marijuana) (Malone & Taylor, 1999) to produce a potent multi-drug cocktail commonly known as “Nyaope” or “Whoonga” (Hull, 2010; Larkan *et al.*, 2010; Cullinan, 2011; Fihlani, 2011; Mokwena & Huma, 2014). Recently, the rewarding and addictive profile of EFV has been established in an animal model, with both sub-acute and sub-chronic exposure to EFV (at a dose of 5 mg/kg) found to induce drug-seeking behaviour in the conditioned place preference (CPP) paradigm in rats (Möller *et al.*, 2018).

EFV is the antiretroviral therapy most commonly associated with neuropsychiatric adverse effects viz. anxiety, acute psychosis, depression and hallucinations (Adkins & Noble, 1998; Puzantian, 2002; Raines *et al.*, 2005; Gatch *et al.*, 2013), although its abuse as a component of the “Nyaope”-cocktail has introduced serious psychosocial issues, including criminality and treatment resistance to anti-HIV medication amongst others. Anxiogenic (at 50 mg/kg) and depressogenic behaviour (at 25 mg/kg and 50 mg/kg) behaviour have earlier been reported following sub-chronic EFV exposure (Cavalcante *et al.*, 2017). The involvement of the central nervous system in mediating these effects could provide insight on the neurochemical mechanisms underlying the bio-behavioural profile of EFV and how this may contribute to “Nyaope”-addiction.

Therefore, the primary aims of this study (in bold) and their associated outcomes can be summarised as follows, and described in more detail in Table 4-1:

1. To investigate the effects of EFV, Δ^9-THC as well as the combination of EFV + Δ^9-THC exposure in rats on social interactive behaviour (utilizing the SIT), anxiety-like behaviour (utilizing the EPM) and sensorimotor gating (utilizing PPI of the acoustic startle reflex), compared to rats only receiving vehicle (pharmaceutical grade olive oil) (Chapter 3).

EFV significantly decreased social interactive behaviour and increased self-directed behaviour in the SIT, when compared to the vehicle control (Table 4-1). Rats exposed to EFV also presented with anxiogenic behaviour in the EPM as well as deficits in sensorimotor gating as indicated by significant reductions in % PPI compared to the vehicle control (Table 4-1). Rats exposed to Δ^9-THC presented with decreased social interaction, although this was not as pronounced as that seen with EFV alone. Δ^9-THC also induced deficits in sensorimotor gating (at 84 dB) compared to the vehicle control (Table 4-1). However, in contrast with that of EFV, rats exposed to Δ^9-THC presented with anxiolytic behaviour in the EPM when compared to the vehicle control (Table 4-1). Rats exposed to the combination of EFV + Δ^9-THC presented with a pro-social behavioural profile, as well as with a psychotogenic-like profile compared to the vehicle control (Table 4-1). However, the combination of EFV + Δ^9-THC had no significant effect on anxiety-like behaviour in the EPM compared to the vehicle control (Table 4-1).

2. To compare the respective exposure groups with each other in order to observe a possible trend in the behavioural profile induced by each drug and to establish whether the drugs act synergistically with one another (Chapter 3).

EFV presented with more pronounced deficits in social interaction than Δ^9-THC, considering that EFV also caused a significant decrease in the time spent anogenital sniffing when compared to Δ^9-THC alone (Table 4-1). Comparing EFV to the combination of EFV + Δ^9-THC, we observed a significant decrease in all pro-social behavioural parameters studied as well as a significant increase in non-social exploratory behaviour (Table 4-1). The pro-social behavioural profile of the combination of EFV + Δ^9-THC were highlighted again when we compared Δ^9-THC alone to the combination of EFV + Δ^9-THC, finding a significant increase in non-social investigatory behaviour (Table 4-1).

EFV also presented with a pronounced anxiogenic profile when compared to Δ^9-THC (Table 4-1). The anxiolytic properties of Δ^9-THC were emphasized when compared to the combination of EFV + Δ^9-THC (Table 4-1).

Comparing EFV and Δ^9-THC alone to the combination of EFV + Δ^9-THC, we observed a significant decrease in sensorimotor gating for Δ^9-THC, thus ascribing a more pronounced psychotogenic behavioural profile to the combination of EFV + Δ^9-THC (Table 4-1).

Table 4-1: Summary of the behavioural analysis in adolescent male Sprague-Dawley (150-180g; n = 18 per group) rats exposed to alternating drug-vehicle administration of vehicle (pharmaceutical grade olive oil), EFV (5 mg/kg), Δ^9-THC (0.75 mg/kg) and the combination of EFV + Δ^9-THC (5 mg/kg and 0.75 mg/kg) for 17 days. Social interactive and self-directed behaviour were scored in the social interaction test (SIT), anxiolytic and anxiogenic behaviour in the elevated plus maze (EPM) and % pre-pulse inhibition in the pre-pulse inhibition (PPI) test. ↑ = significant increase; ↓ = significant decrease; - = no significant / noticeable change. Abbreviations: efavirenz (EFV); Δ^9-tetrahydrocannabinol (Δ^9-THC).

Behavioural analysis														
Comparing exposure groups	**SIT**						**EPM**				**PPI**			
	Social interactive behaviour			**Self-directed behaviour**			**Anxiolytic behaviour**		**Anxiogenic behaviour**		**% PPI**			
	Total time spent together	Times approaching each other	Time spent anogenital sniffing	Time spent self-grooming	Time spent rearing	Total distance moved	Entries into open arms	% Time in open arms	Entries into closed arms	% Time in closed arms	72 dB	76 dB	80 dB	84 dB
EFV vs. Vehicle	-	-	↓	↑	↑	-	-	↓	-	↑	↓	↓	-	-
Δ^9-THC vs. Vehicle	-	-	-	↑	↑	-	↑	-	↓	↓	-	-	-	↓
EFV + Δ^9-THC vs. Vehicle	↑	↑	-	-	-	↓	-	-	-	-	↓	↓	↓	↓

EFV vs. Δ^9-THC	-	-	↓	-	-	-	↓	↓	-	↑	-	-	-	-
EFV vs. EFV + Δ^9-THC	↓	↓	↓	-	↑	↑	-	-	-	-	↑	-	-	↑
Δ^9-THC vs. EFV + Δ^9-THC	-	-	-	-	↑	↑	↑	-	↓	-	↑	↑	-	-

Moreover, we also established that EFV and Δ^9-THC do not act synergistically when combined, with the combination of EFV + Δ^9-THC evoking a unique behavioural profile that did not resemble that produced by these drugs individually.

Subsequently we considered whether the above behavioural alterations could be attributed to drug-induced neuroendocrine- and immune-inflammatory alterations. Since disturbances in tryptophan metabolism have previously been linked to the manifestation of psychiatric illnesses such as depression (Myint *et al.*, 2012) and psychosis (Muller *et al.*, 2011; Erhardt *et al.*, 2017), we investigated the tryptophan-kynurenine pathway as a possible contributor to drug-induced neuroinflammation in this model.

Therefore, the secondary aims secondary aims (in bold) of the study (in bold) and their associated outcomes can be summarised as follows, and described in more detail in Table 4-2:

1. To investigate whether altered behaviours (if any) in EFV, Δ^9-THC and EFV + Δ^9-THC exposed rats are associated with altered oxytocin (OT)- and plasma pro- and anti-inflammatory cytokine levels as well as altered tryptophan-kynurenine metabolism compared to rats only exposed to vehicle (Chapter 3).

The combination of EFV + Δ^9-THC induced a significant increase in the levels of the 'pro-social' peptide hormone, OT in the hippocampus, when compared to the vehicle control group (Table 4-2). Further, rats exposed to EFV had increased plasma levels of the pro-inflammatory cytokine, TNF-α compared to the vehicle control group (Table 4-2). EFV exposure increased levels of the neurodegenerative *N*-methyl-*D*-aspartate (NMDA) receptor agonist, QA and decreased levels of the neuroprotective NMDA receptor antagonist, KYNA compared to the vehicle control (Table 4-2). On the other hand, Δ^9-THC exposure decreased plasma levels of the anti-inflammatory cytokine, IL-10, in comparison with the vehicle control group (Table 4-2), thus suggesting a common pro-inflammatory outcome for both EFV and Δ^9-THC albeit by different means, viz. increasing pro- and decreasing anti-inflammatory cytokine levels, respectively (Table 4-2). The combination of EFV + Δ^9-THC did not cause a significant alteration in the specific pro- or anti-inflammatory cytokine levels, yet still shifted tryptophan metabolism towards the kynurenine pathway. When compared to the vehicle control group, the combination of EFV + Δ^9-THC significantly increased plasma levels of QA and significantly decreased plasma levels of KYNA (Table 4-2). Consequently, EFV, Δ^9-THC and the combination of EFV + Δ^9-THC significantly decreased the neuroprotective ratio compared to the vehicle control group (Table 4-2).

2. To compare the neuroendocrine and immune-inflammatory alterations induced by EFV, Δ^9-THC and EFV + Δ^9-THC exposure with each other in order to observe any trends in the mechanisms by which these drugs possible produce behavioural alterations (Chapter 3).

The anti-social behavioural profile of EFV and Δ^9-THC alone was supported by their ability to significantly decrease hippocampal OT levels when compared to the combination of EFV + Δ^9-THC (Table 4-2).

Whem compared to Δ^9-THC, EFV also caused a significant increase in plasma IL-10 levels (Table 4-2) again emphasizing the deficits in the anti-inflammatory response produced by Δ^9-THC. The pro-inflammatory properties of EFV were highlighted by its ability to cause a significant increase in plasma TNF-α levels when compared to the combination of EFV + Δ^9-THC (Table 4-2).

Table 4-2: Summary of the peripheral and neurochemical analysis in adolescent male Sprague-Dawley rats (150-180g; n = 18 per group) exposed to alternating drug-vehicle administration of vehicle (pharmaceutical grade olive oil), EFV (5 mg/kg), Δ^9-THC (0.75 mg/kg) and the combination of EFV + Δ^9-THC (5 mg/kg and 0.75 mg/kg) for 17 days. Pro- and anti-inflammatory cytokines levels (TNF-α; IL-10) as well as kynurenine pathway metabolites (tryptophan; kynurenine; KYNA; QA; neuroprotective ratio) were measured in the plasma. The concentration of oxytocin levels was measured in the hippocampus. ↑ = significant increase; ↓ = significant decrease; - = no significant / noticeable change. Abbreviations: efavirenz (EFV); Δ^9-tetrahydrocannabinol (Δ^9-THC); tumor necrosis factor alpha (TNF-α); interleukin-10 (L-10); kynurenic acid (KYNA); quinolinic acid (QA); oxytocin (OT).

Peripheral and neurochemical analysis								
Comparing exposure groups	**Peripheral analysis**							**Neurochemical analysis**
	Plasma cytokine levels		**Plasma levels of kynurenine metabolites and the neuroprotective ratio**					Hippocampal OT levels
	Pro-inflammatory TNF-α	Anti-inflammatory IL-10	Tryptophan	Kynurenine	KYNA	QA	Neuroprotective ratio	
EFV vs. Vehicle	↑	-	-	-	↓	↑	↓	-
Δ^9-THC vs. Vehicle	-	↓	↓	-	↓	↑	↓	-
EFV + Δ^9-THC vs. Vehicle	-	-	-	-	↓	↑	↓	↑

EFV vs. Δ^9-THC	-	↑	-	-	-	-	-	-
EFV vs. EFV + Δ^9-THC	↑	-	-	-	-	-	-	↓
Δ^9-THC vs. EFV + Δ^9-THC	-	-	↓	-	-	-	-	↓

4.2 Recommendations

Although this study has provided significant evidence for neuroendocrine and immune-inflammatory mechanisms in mediating the behavioural effects induced by EFV alone or in combination with Δ^9-THC (as in "Nyaope"), several uncertainties and inexplicable findings remain that need clarification in future studies. These are discussed below:

- The behavioural profile induced by the combination of EFV + Δ^9-THC differs from that induced by EFV and Δ^9-THC separately. Further investigation into the mechanism of action as well as the pharmacokinetic- and inflammatory properties of the combination of EFV + Δ^9-THC could provide insight on this observation, especially in explaining how this may relate to the behavioural profile of the "Nyaope"-cocktail which may contain both these drugs and in differing ratios (see next point).

- The exact composition of a "Nyaope"-cocktail has not yet been determined since it appears that the ingredients differ significantly depending on the maker and area where the cocktail is prepared and sold. However, there seems to be a consensus between media reports that most "Nyaope"-cocktails contain antiretroviral drugs, especially EFV (Sciutto, 2009; Cullinan, 2011; Fihlani, 2011). However, other illicit substances such as heroin, marijuana and methamphetamine have also been included in "Nyaope" (Hull, 2010; Larkan *et al.*, 2010; Cullinan, 2011; Michel, 2012). While the behavioural profile of the combination of EFV + Δ^9-THC has been investigated in this study, this may not necessarily reflect that of the "Nyaope"-cocktail. Indeed, the interaction between EFV and methamphetamine and / or heroin and its effect on behaviour has yet to be studied. Thus, further investigation on the composition of "Nyaope" and the behavioural and neurochemical effects of the combination of all these drugs will possibly provide a behavioural profile that accurately resembles the clinical manifestations of "Nyaope" addicts.

- The behavioural profiles of EFV, Δ^9-THC and the combination of EFV + Δ^9-THC could, in part, be attributed to the emergence of a pro-inflammatory state due to increased levels of pro-inflammatory cytokines (such as TNF-α). Thus, research into anti-inflammatory treatment (such as minocycline (Krady *et al.*, 2005)) could be beneficial in countering the effects produced by these drugs.

- EFV, Δ^9-THC as well as the combination of EFV + Δ^9-THC activated the kynurenine pathway. We have attributed the activation of this pathway by EFV and Δ^9-THC alone to

an increase in pro-inflammatory TNF-α and decrease in anti-inflammatory IL-10, respectively. However, the combination of EFV + Δ^9-THC did not have a significant effect on these specific pro- or anti-inflammatory cytokines measured in this study, yet activation of the kynurenine pathway was still evident. This suggests an alternative mechanism of the combination of EFV + Δ^9-THC in inducing a pro-inflammatory state. Pro-inflammatory cytokines such as interleukin-2 (IL-2) and interferon alpha (IFN-α) directly increase indoleamine-2,3-dioxygenase (IDO) enzyme activity which shifts tryptophan metabolism towards the kynurenine pathway (Capuron & Miller, 2011). Thus, determining plasma concentrations of these pro-inflammatory cytokines and other anti-inflammatory cytokines (such as IL--4) could be of value in determining the possible mechanism/s by which the kynurenine pathway is activated by EFV + Δ^9-THC Moreover, inhibitors of this pathway [e.g. 1-methyl-*D*-tryptophan (inhibitor of IDO), allopurinol (inhibitor of TDO) and 3,4-dimethoxy-*N*-[4-(3-nitrophenylthiazol-2-yl] benzenesulfonamide (Ro 61-8048) (inhibitor of kynurenine-3-monooxygenase) (Réus *et al.*, 2018)] could have substantial value in providing, neuroprotection and research in this regard is needed.

- Serotonin is significantly involved in the genesis of anxiety (Millan, 2003). Inhibition of tryptophan metabolism via the kynurenine pathway (which reduces serotonin production and may underlie the manifestation of anxiety responses) could serve as possible treatment for anxiety-like responses in this regard.

- We investigated the effects of a rewarding dose of EFV, Δ^9-THC and the combination of EFV + Δ^9-THC on the behavioural profile of adolescent rats. Investigation into the long-lasting neuroadaptive effects of adolescent drug-exposure (to these specific drugs) on adulthood could provide insight on the neurotoxic and neurodegenerative properties of these drugs.

4.3 Novel findings and conclusion

This study demonstrated that sub-chronic EFV (5 mg/kg), Δ^9-THC (0.75 mg/kg) and the combination of EFV + Δ^9-THC induced significant alterations in social- and anxiety-like behaviour as well as sensorimotor gating. All these drugs caused significant deficits in sensorimotor gating, indicative of a psychotic-like behavioural profile, as discussed in Chapter 3. EFV and Δ^9-THC alone decreased social interactive behaviour, whilst the combination of EFV + Δ^9-THC promoted social interaction. Other known drugs of abuse viz. opiates are also known to increase social interaction albeit still possessing an addictive-profile (Blanco-Gandía *et al.*, 2015). EFV also induced anxiogenic behaviour whilst Δ^9-THC alone induced anxiolytic behaviour. The effects of

addictive drugs such as Δ^9-THC on anxiety may vary depending on the dose used, with high dosages inducing anxiogenic behaviour and low doses inducing anxiolytic behaviour (Rubino *et al.*, 2015). Therefore, this study established that the recreational use of EFV alone or in combination with other known drugs of abuse such as Δ^9-THC (the latter reported to be present in "Nyaope") will present with a distinct behavioural profile which may include social deficits, psychotogenic and anxiogenic behaviour.

This study also provided insight on some of the mechanisms responsible for mediating the above-mentioned drug-induced behavioural effects. Neuroinflammation caused by increases in pro-inflammatory cytokines and decreases in anti-inflammatory cytokines appear to play a central role in mediating the neurotoxic effects the drugs evaluated in the study, since EFV, Δ^9-THC and the combination of EFV + Δ^9-THC activated the tryptophan-kynurenine pathway which lead to an increase in neurodegenerative metabolites and decreased neuroprotective metabolites. However, it seems that other immune-inflammatory mechanisms (such as increased levels of other pro-inflammatory cytokines and decreased levels of anti-inflammatory cytokines not measured in this study) are involved in shifting tryptophan metabolism via the kynurenine pathway when EFV is used in combination with Δ^9-THC. However, further investigation in this regard is needed.

This study succeeded in evaluating the bio-behavioural effects of EFV exposure alone or in combination with a known drug of abuse, such as Δ^9-THC, with regards to social interactive and anxiety-like behaviour as well as sensorimotor domains. Moreover, this study successfully attributed possible underlying neuroendocrine / immune-inflammatory mechanisms to the emergence of an addictive bio-behavioural profile that may possibly resemble that of "Nyaope"-addicts.

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ADDENDUM A

Enzyme-Linked-Immunosorbent Assay (ELISA) kits

Aims

To create standard calibration curves for plasma pro-inflammatory (tumor necrosis factor alpha (TNF-α)) and anti-inflammatory (interleukin-10 (IL-10)) cytokines as well as hippocampal oxytocin (OT), in order to calculate the concentration of each cytokine in the plasma and OT levels in the hippocampus of rats exposed to vehicle (pharmaceutical grade olive oil), efavirenz (EFV), Δ^9-tetrahydrocannabinol (Δ^9-THC) as well as the combination of EFV + Δ^9-THC as evaluated in Chapter 3.

A1 Quantification of rat plasma TNF-α levels

A1.1 Introduction

An increase in pro-inflammatory cytokines (such as TNF-α) have been linked to the emergence of a pro-inflammatory state (Clark *et al.*, 2013), since TNF-α can exert inflammatory and cytotoxic effects on several normal lymphoid and non-lymphoid cells. BioLegend's ELISA MAX™ Deluxe kits were used for the quantification of rat TNF-α plasma levels.

A1.2 Materials

- Rat TNF-α ELISA Capture Antibody (200X)
- Rat TNF-α ELISA Detection Antibody (200X)
- Rat TNF-α Standard
- Avidin-Horseradish Peroxidase (HRP) (1,000X)
- Matrix Diluent A
- Substrate Solution A
- Substrate Solution B
- Coating Buffer A (5X)
- Assay Diluent A (5X)
- NUNC Maxisorp™ 96 MicroWell Plates
- ELISA MAX™ Deluxe Set Protocol
- Phosphate-Buffered Saline (PBS)
- Wash Buffer: PBS + 0.05% Tween-20
- Stop Solution: 2N H_2SO_4

- Plate Sealers: BioLegend
- Deionized water
- Microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes and pipette tips
- Automated microplate washer
- Plate shaker

A1.3 Sample collection

After euthanization, trunk blood was collected in pre-chilled Vacuette® tubes containing a K_2EDTA solution as anti-coagulant and centrifuged (14 000 rotations per minute for 10 minutes at 4°C) within 30 minutes from collection. The supernatant (plasma) was then collected and stored at -80°C until the day of analysis.

A1.4 Reagent preparation

PBS was prepared by dissolving 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl in 1 L deionized water; pH 7.4, 0.2 μm filtered.

1. Coating Buffer A (5X) were diluted to 1X with deionized water by diluting 2.4 ml 5X Coating Buffer A in 9.6 ml deionized water.
2. Pre-titrated Capture Antibody (60 μl)was diluted in 11.94 ml 1X Coating Buffer A.
3. Assay Diluent A (5X) was diluted to 1X with 1X PBS by diluting 10 ml 5X Assay Diluent A n 40 ml 1X PBS.
4. The lyophilized standard was reconstituted with 0.2 ml of 1X Assay Diluent A and allowed to sit for 15 minutes at room temperature and vortexed.
5. The top standard (concentration: 1000 pg/ml) (1 ml) from the stock solution was prepared in 1X Assay Diluent A.
6. Pre-titrated Biotinylated Detection Antibody (60 μl) was diluted in 11.94 ml 1X Assay Diluent A.
7. Avidin-HRP (12 μl) was diluted in 11.99 ml 1X Assay Diluent A.
8. Prior to use, 5.5 ml Substrate Solution A was immediately mixed with 5.5 ml of Substrate Solution B in a clean container to produce TMB Substrate Solution.

A1.5 Assay procedure

1. One day prior to analysis, the wells of a 96-well plate were coated with 100 μl diluted Capture Antibody and incubated overnight (16-18 hours), between 2°C - 8°C.

2. On the day of analysis, the plasma samples were allowed to be thawed on ice and all reagents were brought to room temperature.
3. The plate was washed 4 times with 300 μl wash buffer.
4. 1X Assay Diluent A (200 μl) was added per well.
5. The plate was sealed and incubated at room temperature for 1 hour with shaking on a plate shaker.
6. The plate was washed 4 times with wash buffer.
7. Matrix Diluent A (50 μl) was added to the "standard" wells and 50 μl Assay Diluent A to the "sample" wells.
8. Then, 50 μl standards were added to the "standard" wells or 50 μl sample to the "sample" wells.
9. The plate was sealed and incubated at room temperature for 2 hours with shaking.
10. The plate was washed 4 times with wash buffer.
11. Diluted Detection Antibody (100 μl) was added to each well.
12. The plate was sealed and incubated at room temperature for 1 hour with shaking.
13. The plate was washed 4 times with wash buffer.
14. Avidin-HRP (100 μl) was added to each well.
15. The plate was sealed and incubated at room temperature for 30 minutes with shaking.
16. The plate was washed 5 times with wash buffer. Each wash lasted 30 – 60 seconds.
17. TMB Substrate Solution (100 μl) was added to each well. Positive wells turned blue in colour.
18. The plate was sealed and incubated in the dark at room temperature for 25 minutes
19. Stop Solution was added to each well (100 μl). Positive wells turned from blue to yellow.
20. The absorbance was read at 450 nm and 570 nm.

A1.6 Results

GraphPad Prism was used to plot a linear standard curve (Fig. A1-1) and a four-parameter logistic curve fit (Fig A1-2). Rat plassma TNF-α showed a linear regression of 0.999, with a formula of y = 1*X – 0.0514 (Fig. A1-1).

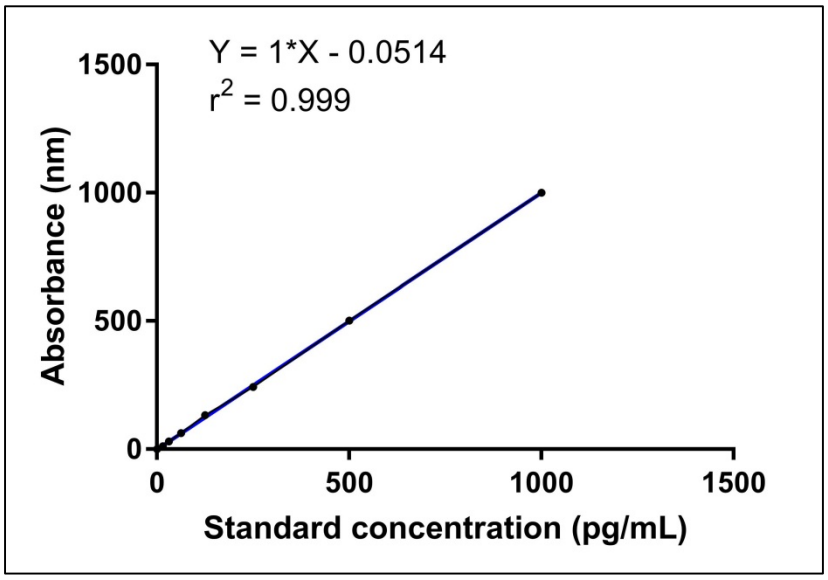

Fig. A1-1: Standard calibration curve for rat plasma TNF-α, determined by ELISA kits.

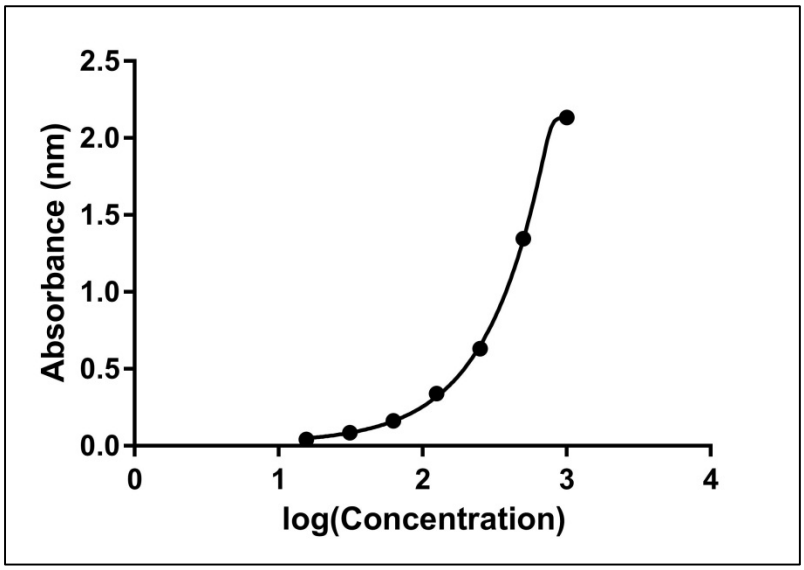

Fig. A1-2: Logistic curve for rat plasma TNF-α, determined by ELISA kits.

A1.7 Conclusion

The discussed methods for quantification of plasma TNF-α levels were applied in the current study as described in detail in Chapter 3.

A2 Quantification of rat plasma IL-10 levels

A2.1 Introduction

Immunoregulatory anti-inflammatory cytokines (such as IL-10) can inhibit or control the inflammatory process produced by pro-inflammatory cytokines (Cassatella *et al.*, 1993). Elabscience® ELISA kits were used for the quantification of rat IL-10 concentrations in plasma.

A2.2 Materials

- Dismountable Micro ELISA Plate
- Reference Standard
- Concentrated Biotinylated Detection Antibody (100X)
- Concentrated HRP Conjugate (100X)
- Reference Standard and Sample Diluent
- Biotinylated Detection Antibody Diluent
- HRP Conjugate Diluent
- Concentrated Wash Buffer (25X)
- Substrate Reagent
- Stop Solution
- Plate Sealer
- Distilled water
- Microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes and pipette tips
- Incubator capable of maintaining 37°C
- Automated microplate washer

A2.3 Sample collection

After euthanization, trunk blood was collected in pre-chilled Vacuette® tubes containing a K_2EDTA solution as anti-coagulant and centrifuged (14 000 rotations per minute for 10 minutes at 4°C) within 30 minutes from collection. The supernatant (plasma) was collected and stored at -80°C until the day of analysis.

A2.4 Reagent preparation

1. All reagents were brought to room temperature.
2. Wash buffer (750 ml) was prepared by diluting 30 ml of concentrated wash buffer with 720 ml distilled water.

3. The standard working solution (2000 pg/ml) was prepared by centrifuging the standard at 10,000×g for 1 minute. Thereafter, 1 ml of Reference Standard and Sample Diluent were added. The mixture was gently inverted several times and allowed to stand for 10 minutes.
4. Biotinylated Detection Antibody working solution (100 μl/well) was prepared by diluting the 100X Concentrated Biotinylated Detection Antibody to 1X working solution with Biotinylated Detection Antibody Diluent.
5. Concentrated HRP Conjugate working solution (100 μl/well) was prepared by diluting the 100X Concentrated HRP Conjugate to 1X working solution with Concentrated HRP Conjugate Diluent.

A2.5 Assay procedure

On the day of analysis, the plasma samples were allowed to be thaw on ice.

1. Standard working solution was added to the first two columns: Each concentration of the solution was added in duplicate, to one well each, side by side (100 μl for each well). The plasma samples were added to the other wells (100 μl for each well).
2. The plate was sealed and incubated for 90 minutes at 37°C.
3. The liquid was removed out of each well, without washing. Then, 100 μl of Biotinylated Detection Antibody working solution was immediately added to each well.
4. The plate was sealed and incubated for 1 hour at 37°C.
5. The solution was decanted from each well and 350 μl of wash buffer was added to each well. The plate was then soaked for 1~2 minutes and then the solution was decanted from each well. Thereafter, the plate was pat dry against clean absorbent paper and this wash step was repeated 3 times.
6. HRP Conjugate working solution (100 μl) was added to each well.
7. The plate was sealed and incubated for 30 minutes at 37°C.
8. The solution was decanted from each well and the wash process described in step 5 was repeated five times.
9. Substrate Reagent (90 μl) was added to each well.
10. The plate was sealed and incubated for about 15 minutes at 37°C.
11. Stop Solution (50 μl) was added to each well in the same order as the substrate solution.
12. The optical density (OD value) of each well was determined with a micro-plate reader set to 450 nm.

A2.6 Results

GraphPad Prism was used to plot a linear standard curve (Fig. A2-1) and a four-parameter logistic curve (Fig. A2-2). Rat plasma IL-10 showed a linear regression of 0.99, with a formula of y = 0.001704*X + 0.02712 (Fig. A2-1).

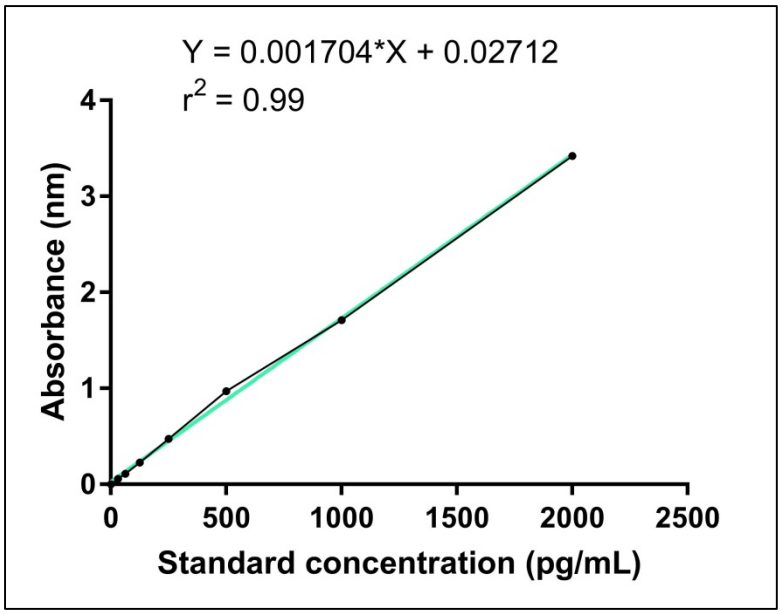

Fig. A2-1: Standard calibration curve of rat plasma IL-10, determined by ELISA kits.

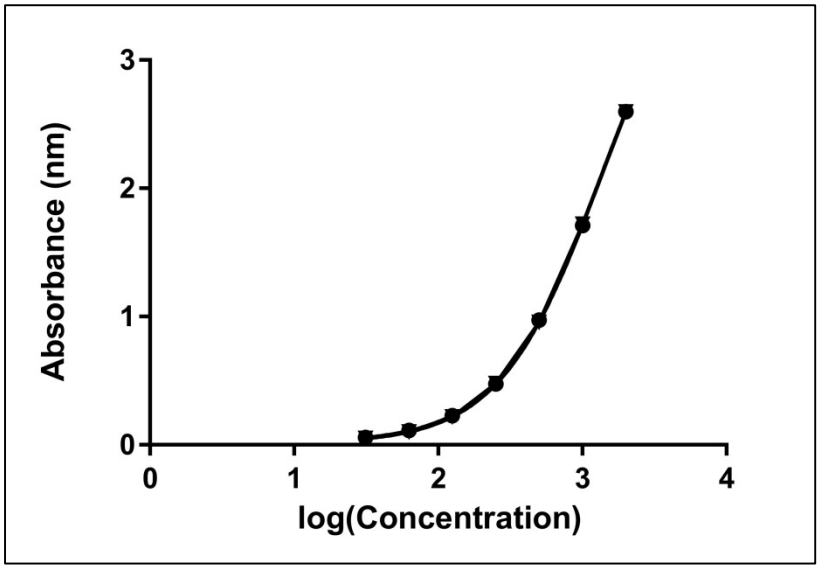

Fig. A2-2: Logistic curve for rat plasma IL-10, determined by ELISA kits.

A2.7 Conclusion

The discussed methods for quantification of plasma IL-10 levels were applied in the current study as described in detail in Chapter 3.

A3 Quantification of rat hippocampal OT levels

A3.1 Introduction

OT is a nine-amino acid peptide hormone implicated to be involved in the mediation of 'pro-social' and anxiolytic behaviour (McRae-Clark *et al.*, 2013). Elabscience® ELISA kits were used for the quantification of rat OT concentrations in the hippocampus.

A3.2 Materials

- Sonicator
- PBS
- Dismountable Micro ELISA Plate
- Reference Standard
- Concentrated Biotinylated Detection Antibody (100X)
- Concentrated HRP Conjugate (100X)
- Reference Standard and Sample Diluent
- Biotinylated Detection Antibody Diluent
- HRP Conjugate Diluent
- Concentrated Wash Buffer (25X)
- Substrate Reagent
- Stop Solution
- Plate Sealer
- Distilled water
- Microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes and pipette tips
- Incubator capable of maintaining 37°C
- Automated microplate washer

A3.3 Sample collection

After euthanization, the hippocampus was identified and carefully dissected, immediately snap frozen in liquid nitrogen and stored at -80°C until the day of analysis.

A3.4 Sample preparation

PBS was prepared by dissolving 200 g KCl and 22.5 g NA_2HPO_4 in 2.5 L distilled water. One part of the PBS solution was diluted with 9 parts distilled water to obtain a 0.01 mM PBS solution.

1. Samples were left to thaw on ice and weighed.
2. Ice-cold PBS (pH 7.4) was used to make a 10% m/v solution with the brain samples, where after they were homogenised by ultrasonification.
3. The samples were then centrifuged for 5 minutes at 5000×g to obtain the supernatant.

A3.5 Reagent preparation

1. All reagents were brought to room temperature.
2. Wash buffer (750 ml) was prepared by diluting 30 ml of concentrated wash buffer with 720 ml distilled water.
3. The standard working solution (1000 pg/ml) was prepared by centrifuging the standard at 10,000×g for 1 minute. Thereafter, 1.0 ml of Reference Standard and Sample Diluent were added. The mixture was gently inverted several times and allowed to stand for 10 minutes.
4. Biotinylated Detection Antibody working solution (50 μl/well) was prepared by diluting the 100X Concentrated Biotinylated Detection Antibody to 1X working solution with Biotinylated Detection Antibody Diluent.
5. Concentrated HRP Conjugate working solution (100 μl/well) was prepared by diluting the 100X Concentrated HRP Conjugate to 1X working solution with Concentrated HRP Conjugate Diluent.

A3.6 Assay procedure

1. Standard working solution was added to the first two columns: Each concentration of the solution was added in duplicate, to one well each, side by side (50 μL for each well). The plasma samples were added to the other wells (50 μL for each well). Immediately thereafter, 50 μL of Biotinylated Detection Antibody working solution was added to each well.
2. The plate was sealed and incubated for 45 minutes at 37°C.
3. The solution was decanted from each well and 350 μL of wash buffer was added to each well. The plate was then soaked for 1~2 minutes and then the solution was decanted from each well. Thereafter, the plate was pat dry against clean absorbent paper and this wash step was repeated 3 times.
4. HRP Conjugate working solution (100 μL) was added to each well.
5. The plate was sealed and incubated for 30 minutes at 37°C.
6. The solution was decanted from each well and the wash process described in step 3 was repeated five times.
7. Substrate Reagent (90 μl) was added to each well.

8. The plate was sealed and incubated for about 15 minutes at 37°C.
9. Stop Solution (50 μl)was added to each well in the same order as the substrate solution.
10. The optical density (OD value) of each well was determined with a micro-plate reader set to 450 nm.

A3.7 Results

GraphPad Prism was used to plot a linear standard curve (Fig. A3-1) and a four-parameter logistic curve (Fig. A3-2). Rat hippocampal OT levels showed a linear regression of 0.99, with a formula of y = 0.01142*X + 0.1276 (Fig. A3-1).

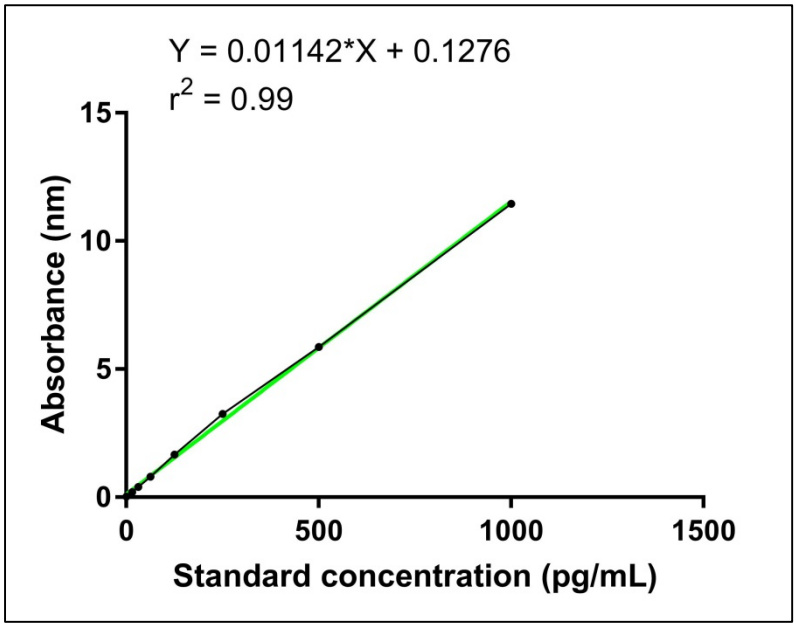

Fig. A3-1: Standard calibraton curve of rat hippocampal OT, determined by ELISA kits.

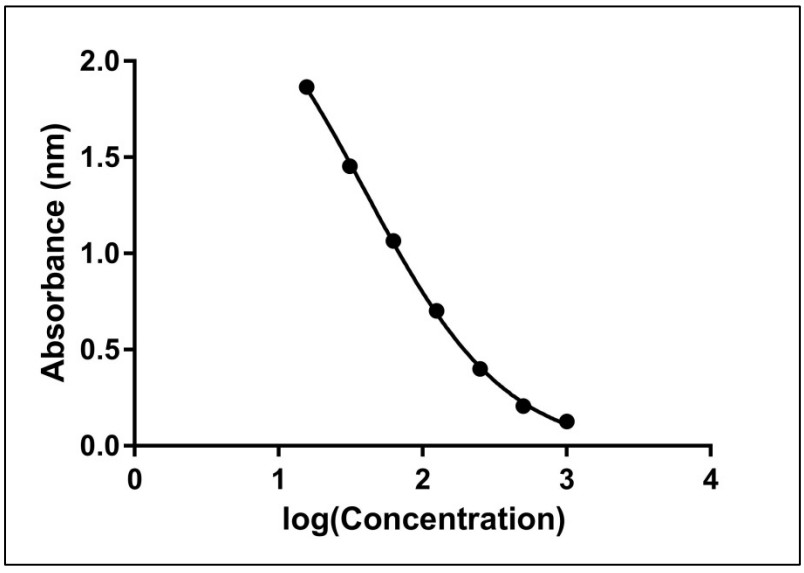

Fig. A3-2: Logistic curve for rat hippocampal OT, determined by ELISA kits.

A3.8 Conclusion

The discussed methods for quantification of hippocampal OT levels were applied in the current study as described in detail in Chapter 3.

ADDENDUM B

Determining tryptophan-metabolites using a high performance liquid chromatography (HPLC) system

B1.1 Introduction

Tryptophan metabolism entails the conversion of tryptophan into serotonin by tryptophan-5-hydroxylase, or into kynurenine by the two principle haemdependant enzymes tryptophan-2,3-dioxygenase (TDO) or indoleamine-2,3-dioxygenase (IDO) (Möller *et al.*, 2015). A pro-inflammatory state will shift tryptophan-metabolism towards the kynurenine pathway by activating TDO or IDO (Möller *et al.*, 2015). Quantification of plasma tryptohan-metabolites (viz. tryptophan, kynurenine, kynurenic acid (KYNA), and quinolinic acid (QA) was determined as described previously (Badawy & Morgan, 2010; Möller *et al.*, 2012).

B1.2 Materials

- Solid phase extraction cartridges
- Methanol (CH_3OH)
- Formic acid (CH_2O_2) (FA)
- Internal standard (Anthranilic acid isopropylamide)
- Distilled water
- Perchloric acid ($HCLO_4$)
- Vortex
- Centrifuge capable of 14 000 rpm
- Extraction column and vacuum dryer
- Ammonium hydroxide (NH_4OH)
- Tetrahydrofuran [$(CH_2)_4O$]
- Evaporating chamber
- Nitrogen (N_2) gas
- HPLC system capable of measuring analytes at a wavelength of 220 nm
- Analytical HPLC column (Venusil ASB C8)

B1.3 Sample collection

After euthanization, trunk blood was collected in pre-chilled Vacuette® tubes containing a K_2EDTA solution as anti-coagulant and centrifuged (14 000 rotations per minute for 10 minutes

at 4°C) within 30 minutes from collection. The supernatant (plasma) was collected and stored at -80°C until the day of analysis.

B1.4 Chromatographic conditions

The following specific parameters (including analytical instruments, column, flowrate, injection volume and wavelength) were set and used to measure the tryptophan-metabolites.

Table B-1: Chromatographic conditions

Analytical instrument	Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem III Electrochemical detector (with Coulometric flow cell) and Chromeleon® Chromatography Management System version 6.8.
Column	The analytical HPLC column used was a Venusil ASB C8 (purchased from Bonna-Agela Technologies, USA), 4.6 x 250 mm, a particle size of 5 µm, pore size of 150 Å and a surface area of 200 m^2/g.
Flow rate	1ml/min
Injection volume	100 μl
Diode Array Detector (DAD) settings	Wavelength was set at 220 nm for all the analytes except Quinolinic acid was monitored at 265nm.
Run time	± 28 minutes

B1.5 Mobile phase preparation

A mobile phase consisting of 0.1 M sodium formate buffer and 24% v/v methanol was prepared. The pH of the mobile phase was set at ± pH 2.05 with ortho-phosphoric acid. The mobile phase was filtered through a 0.22 µm nylon filter before use (Agela Technologies).

B1.6 Standard preparation

The standard solution contains known concentrations of tryptophan and its metabolites. This standard solution was used to determine the unknown concentrations of tryptophan and its metabolites in the plasma samples.

Solution A (used in all sample preparations and standard solutions), the respective stock solutions for all the standards as well as the internal standard was prepared as follows:

Preparation of Solution A:

Solution A was prepared by adding 2 ml methanol to 98 ml distilled water.

Preparation of stock solutions:

Each analyte (1 mg) was dissolved in 10 ml of solution A, which yielded a concentration of 100 μl/ml for each analyte. Stock solutions of tryptophan, kynurenine, kynurenic acid and quinolinic acid were prepared.

From the stock solutions a concentration range of10 - 500 ng/ml standards were prepared in distilled water (see Table B-2) and stored between 2 - 8°C.

Table B-2: Preparation of standard solutions

Standard number	Concentration (ng/ml)	Dilution	+	Solution A	=	Total volume
1	10	4	+	1996	=	2 ml
2	25	10	+	1990	=	2ml
3	50	20	+	1980	=	2 ml
4	75	30	+	1970	=	2 ml
5	100	40	+	1960	=	2 ml

6	150	60	+	1940	=	2 ml
7	500	200	+	1800	=	2 ml

B1.7 Sample preparation

On the day of analysis, plasma samples were allowed to be thaw on ice.

1. While the tubes were kept on ice, the following was added to 800 μl of the plasma samples:
    - 100 μl internal standard
    - 400 μl distilled water
    - 100 μl perchloric acid
2. The samples were voretexed for 10 seconds and allowed to sit for 5 minutes.
3. The samples were centrifuged (14 000 rpm for 10 minutes at 4°C) and the supernatant was collected and kept on ice.
4. The solid phase extraction cartridges (Waters Oasis HLB Extraction Cartridges, Microsep Pty Ltd., Sandton, South Africa) were conditioned with 2 ml CH_3OH and 2 ml 1% CH_2O_2 .
5. The supernatant was loaded onto the extraction column.
6. The extraction cartridges were then washed with 2 ml 1% CH_2O_2.
7. The extraction cartridges were vacuum-dried for 5-10 minutes at ± 20 kPa.
8. Thereafter, the contents of the extraction cartridges were eluted with 1% NH_4OH in CH_3OH: $(CH_2)_4$) and evaporated under a gentle stream of nitrogen in an evaporation chamber.
9. The contents were reconstituted with 125 μl 25% CH_3OH: distilled water.
10. Thereafter, the contents were vortexed for 2 minutes and centrifuged.

B1.8 Calibration and linearity

The linearity of an analytical procedure is the ability to obtain test results in a specific range that is directly proportional to the concentration of the sample. The linearity used in this validation process compromised of the following standard concentrations: 10, 25, 50, 75, 100, 150 and 500 ng/ml for each analyte. The linear regression value determined for each metabolite is presented in figures B-1 – B-4.

Tryptophan showed a linear regression of 0.9857, with a formula of y = 0.0297X + 1.0883

Kynurenine showed a linear regression of 0.9882, with a formula of y = 0.0064X + 0.1149

Kynurenic acid showed a linear regression of 0.987, with a formula of y = 0.0225X – 0.0908

Quinolinic acid showed a liinear regression of 0.9991, with a formula of y = 0.0004X + 0.0144

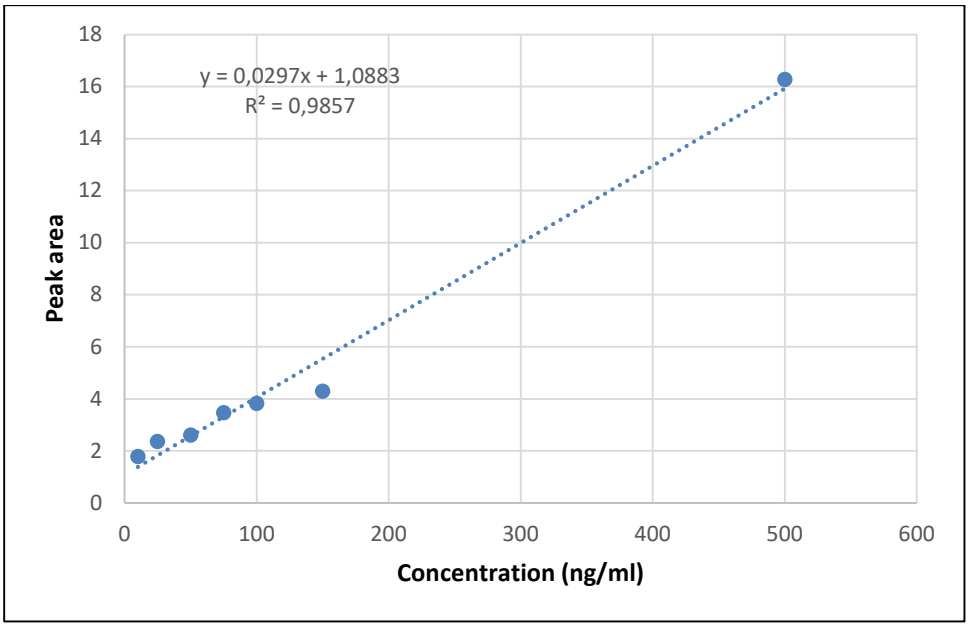

Fig. B-1: Standard calibration curve for tryptophan, determined by HPLC.

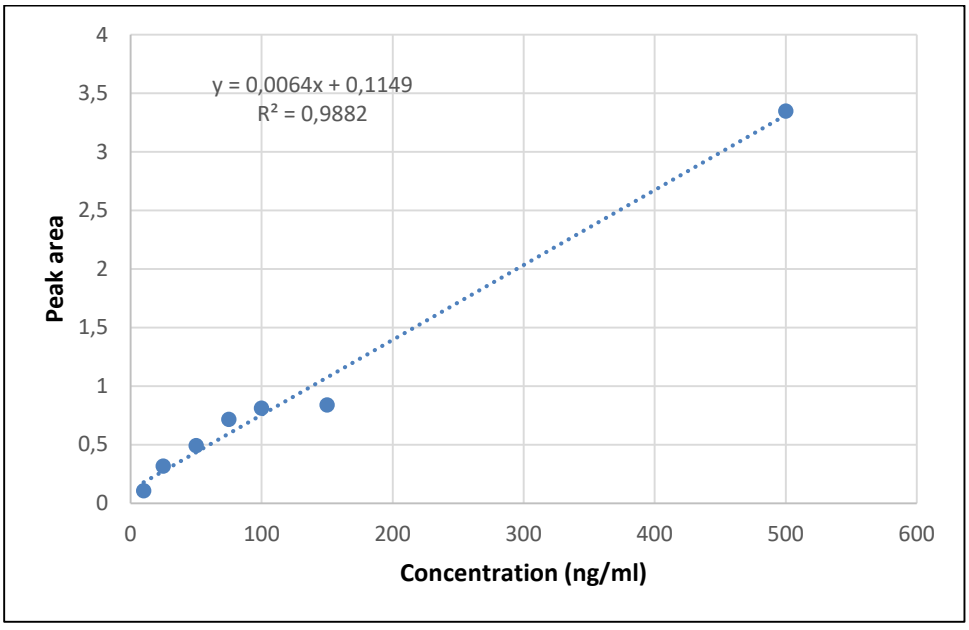

Fig. B-2: Standard calibration curve of kynurenine, determined by HPLC.

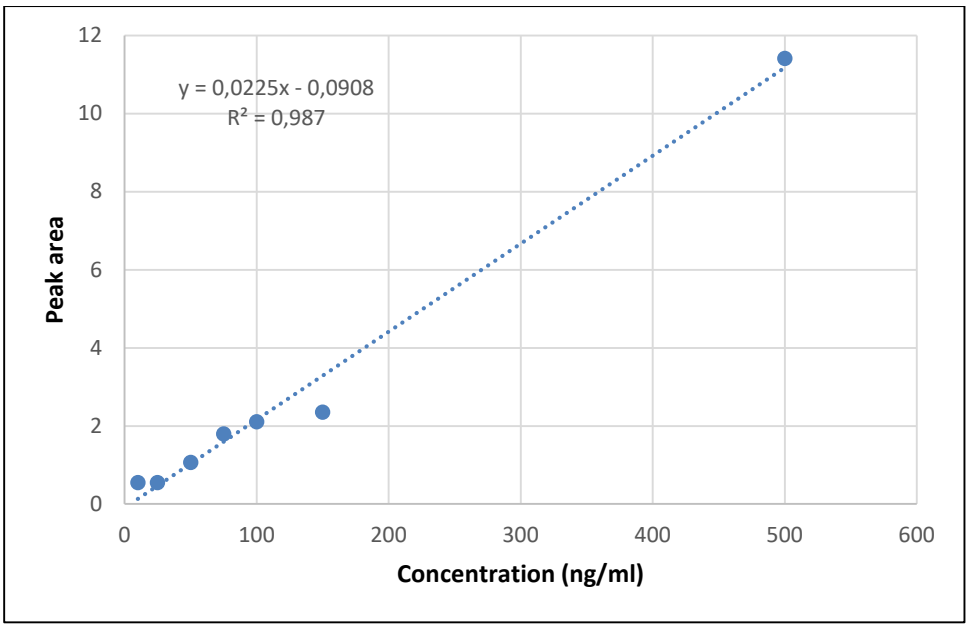

Fig. B-3: Standard calibration curve of kynurenic acid, determined by HPLC.

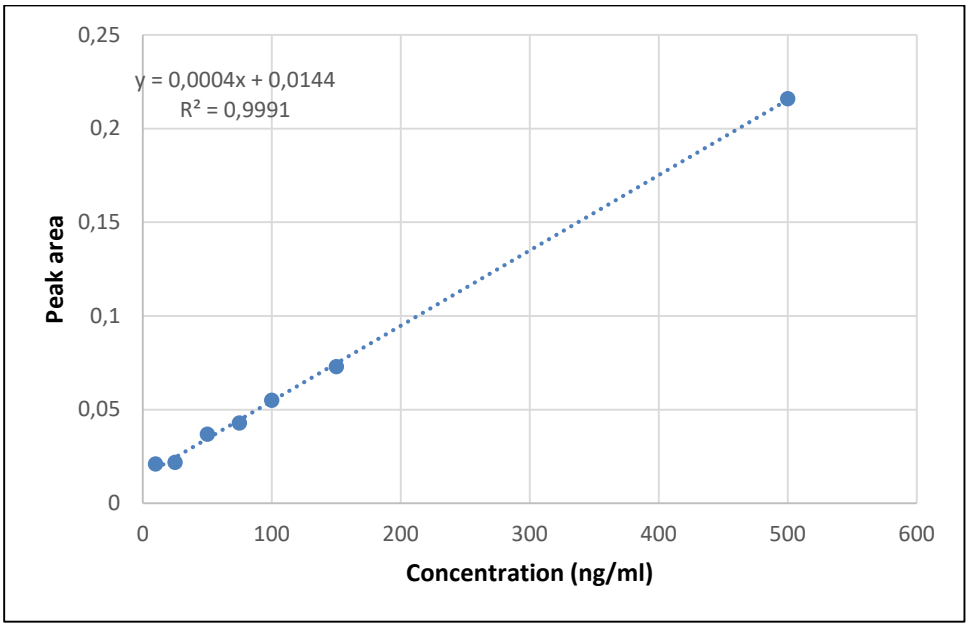

Fig. B-4: Standard calibration curve of quinolinic acid, determined by HPLC.

B1.9 Chromatographic results

The selectivity and specificity refer to the capability of a specific method to accurately analyse specific components in the presence of other components (viz. metabolites and other biological substances). The chromatogram of a plasma sample containing tryptophan, kynurenine, kynurenic acid, quinolinic acid and the internal standard (anthranilic acid isopropylamide) is indicated in Fig. B-5.

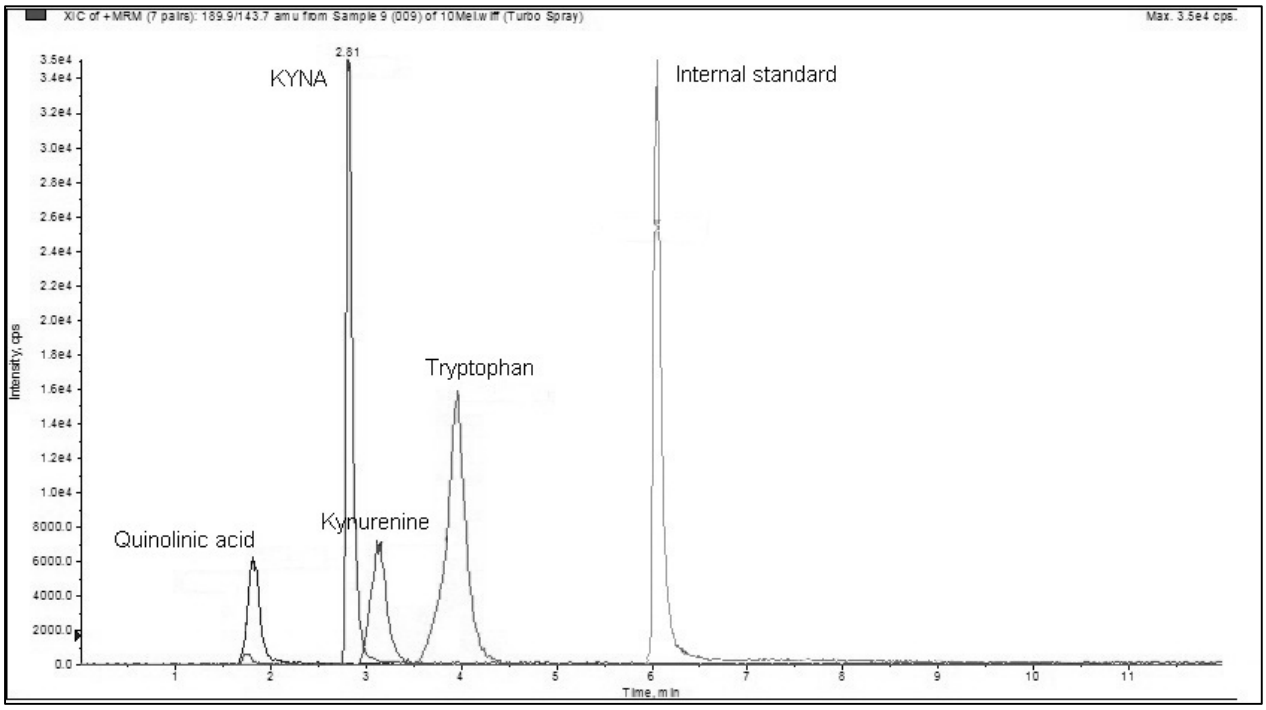

Fig. B-5: Chromatogram of a plasma sample spiked with tryptophan, kynurenine, kynurenic acid (KYNA), quinolinic acid and internal standard, measured in mAU with a retention time of ± 28 minutes.

B1.10 Conclusion

The described methods for the quantification of plasma tryptophan, kynurenine, kynurenic acid and quinolinic acid were applied in the current study as described in detail in Chapter 3.

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ADDENDUM C

30 October 2018

Dear examiner

M.Sc. DISSERTATION – N. MULLER

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. Nadia Muller, I hereby approve that the concept manuscript listed below be included as part of the requirements for fulfilment of the M.Sc. degree, and that this manuscript may be submitted for evaluation purposes by the candidate.

The article is as follows:

Chapter 3

Exposure to efavirenz alone or in combination with Δ^9-tetrahydrocannabinol induces selected bio-behavioural changes that define its abuse potential

Sincerely

Marisa Möller, PhD

30 October 2018

Dear examiner

M.Sc. DISSERTATION – N. MULLER

PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

As co-study leader and co-author on the article presented in Chapter 3, first authored by Ms. Nadia Muller, I hereby approve that the concept manuscript listed below be included as part of the requirements for fulfilment of the M.Sc. degree, and that this manuscript may be submitted for evaluation purposes by the candidate.

The article is as follows:

Chapter 3

Exposure to efavirenz alone or in combination with Δ^9-tetrahydrocannabinol induces selected bio-behavioural changes that define its abuse potential

Sincerely

Brian H. Harvey, PhD