

Evaluation of efavirenz on neurochemical and oxidative stress markers and addictive-like behaviours in rats

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Abstract

HIV positive patients treated with the antiretroviral (ARV) drug efavirenz have been observed to experience various neuropsychiatric symptoms including insomnia, dizziness, somnolence, vivid and abnormal dreams and thoughts and manic episodes, clearly indicating central nervous system (CNS) effects. Moreover, recent news reports in South Africa describe people recreationally using efavirenz by crushing and smoking this ARV drug in a mixture of drugs known as “Nyaope” or “Whoonga”. Other than efavirenz as the main constituent, this mixture is known to contain marijuana, milk powder, rat poison, amongst others. The abuse of this drug places tremendous pressure on the Department of Health and endangers the lives of people in need of ARV treatment. A previous preclinical study suggests that the effects of efavirenz are in line with that of lysergic acid diethylamide (LSD), mediated by a partial agonist effect on the serotonin (5-HT) - $2A$ receptor subtype, the primary binding site for drugs of abuse known as the hallucinogens. Drugs of abuse are known to alter the behaviour and neurochemistry of the abuser and some studies also suggest alterations in the anti-oxidant system of the body, linked to monoamine alterations, in this instance, dopamine (DA), 5-HT and noradrenaline. Animal models enable research to screen for alterations in the above-mentioned indicators and to determine the underlying mechanism through which a drug may elicit its abuse potential and related addictive-like effects. A well-known behavioural test to assess rewarding properties of drugs is the conditioned place preference (CPP) test in which the motivational or aversive properties of a drug serves as an unconditioned stimulus that is continuously paired with an environment. Through Pavlovian principles, this leads to an association of the previously neutral environment with the properties of the drug. Rewarding or addictive drugs are known to significantly increase the time spent in the drug-paired compartment after conditioning. Various other behavioural screening tests such as the sucrose preference test (SPT) may indicate whether a drug interacts with the reward pathways under the control of DAergic signalling, leading to changes in sucrose consumption (as hedonic measure). Moreover, the psychomotor stimulant theory suggests that drugs of abuse induce hyper-locomotion (in the open field test (OFT)) in animals, which is considered a valuable test to screen for drugs of abuse. Furthermore, this test may be of value in supporting the outcome of other tests (such as the CPP test) in which locomotion plays an imperative role. This study aimed to assess the addictive-like properties of efavirenz after sub-acute (6 days) and sub-chronic (14 days) exposure in rats. The present study (ethical approval no: NWU - 00267- 16- S5) used a total of 84 male Sprague Dawley rats (randomly allocated to 7 groups of rats with 12 rats per drug exposure group), bred and housed in the DST/NWU PCDDP Vivarium. The sub-acute paradigm exposed 5 groups of rats to i.p injections of either

5, 10 or 20 mg/kg/d efavirenz, 1 mg/kg/d methamphetamine (MA) (as a positive control) or vehicle in order to establish the most rewarding dosage of efavirenz. CPP, sucrose preference and locomotor activity were assessed in the sub-chronic study, evaluating the effects of efavirenz on reward (CPP), anhedonic manifestations over time (SPT) and locomotion (OFT). In the sub-acute study, MA was compared to a vehicle in order to validate the CPP paradigm under our laboratory conditions. The sub-chronic study was conducted using the most rewarding dose of efavirenz as determined in the sub-acute phase (5mg/kg/d alternate days x 14 days). Furthermore, quantification of frontal cortical, striatal and hippocampal DA, 5-HT, and their respective metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-Hydroxyindoleacetic acid (5-HIAA) as well as NA was performed in the sub-chronic efavirenz exposure study, using a high-performance liquid chromatography system with electrochemical detection (HPLC-EC). Regional brain lipid peroxidation and plasma superoxide dismutase (SOD) levels were also assessed after sub-chronic exposure to efavirenz, utilizing ELISA kits. One-way ANOVA with Dunnett's post hoc test or unpaired student t-tests were applied for statistical analyses with $p < 0.05$ deemed significant. In the sub-acute study, 5 mg/kg efavirenz induced a significant increase in the time spent in the drug-paired compartment compared to the control group in the CPP test. These results were comparable to the rewarding effects of MA in the same test. Efavirenz at 10 mg/kg showed no changes, while 20 mg/kg showed a significant decrease in the time spent in the drug-paired compartment in comparison to the control group. In contrast to data described in the CPP test, no changes in locomotion or sucrose preference were observed. Neurochemical analyses in the sub-chronically exposed efavirenz animals indicated a significant increase in frontal cortical DA and 5-HT levels whilst 5 HIAA levels were significantly lower compared to vehicle exposed animals. DA and 5-HT turnover was significantly decreased in animals exposed to sub-chronic efavirenz compared to a vehicle control. Striatal DA, 5-HT and NA were increased while DOPAC levels and DA turnover was decreased in these animals compared to the vehicle control. Hippocampal DA, DOPAC, 5-HIAA and 5-HT turnover was significantly decreased whereas DA turnover rate was significantly increased in animals exposed to efavirenz compared to the vehicle control. Peripheral (plasma) SOD levels were significantly increased in efavirenz treated animals, while regional brain lipid peroxidation was significantly elevated in the DA rich areas (frontal cortex and striatum), compared to their vehicle control. The findings in the sub-acute and sub-chronic study demonstrate a significant dose dependant rewarding effect of efavirenz in rats (in a validated CPP paradigm under our laboratory conditions), with lower doses being most effective and higher doses eliciting aversive responses. The unaltered preference for sucrose after sub-chronic efavirenz exposure further supports the outcomes that the lower doses are rewarding since no anhedonic behaviour manifested over the period of treatment. Unaltered locomotion suggests that sub-chronic efavirenz does not elicit its

effects through the same striatal-mediated mechanism as do many other drugs of abuse but supports the outcome in the CPP test. Moreover, this implies that performance in the CPP was not a result of undue effects of the drug or condition on the mobility of the animals. The findings obtained in the sub-chronic study confirm that efavirenz alters regional brain DA, 5-HT, NA and lipid peroxidation as well as peripheral SOD levels. This animal study has significant relevance in elucidating the possible mechanism through which efavirenz induces a rewarding effect, which in turn may underlie its abuse potential.

Keywords: efavirenz; nyaope; whoonga; conditioned place preference; drug abuse, monoamines, oxidative stress.

Opsomming

Pasiënte met menslike immuuniteitsgebreksvirus (MIV) wat behandel word met die antiretrovirale middel, efavirenz, ervaar verskeie neuropsigiatriese simptome soos duiseligheid, slapeloosheid, nagmerries en maniese episodes wat kenmerkend van sentraal senuweestelseffekte is. Die misbruik van efavirenz om ontspanning te bewerkstellig is reeds geruime tyd in die nuus; hierdie middel word as deel van 'n mengsel op straat gebruik as "Nyaope" of "Whoonga". Genoemde mengsel bestaan ondermeer uit marijuana (dagga), rottegif, heroïen en poeiermelk. Die misbruik van efavirenz plaas geweldige druk op die departement van gesondheid en stel eweneens die lewens van die pasiënte wat hierdie middel nodig het, in gevaar. 'n Vorige pre-kliniese studie het getoon dat die effekte van efavirenz ooreenstem met dié van die bekende hallusinogeen, D-lisergiensuur-diëtielamied (LSD). LSD se hallusinogeniese effekte word bemiddel deur binding aan die serotonien (5-HT)_{2A} reseptorsub tipe. Dwelmmiddels is bekend daarvoor om die gedrag en neurochemie van die gebruiker te beïnvloed en die resultate van sommige studies suggereer dat dwelmmiddels belangrike veranderinge in die anti-oksidadant sisteem teweeg mag bring wat verband hou met 'n verandering in monoamienergiese neurotransmissie (nl. dopamien (DA), 5-HT en noradrenalin (NA)). Dieremodelle maak dit moontlik om ondersoek in te stel na die wyse waarop sekere dwelmmiddels die bogenoemde veranderinge ontlok en gedragsveranderinge meebring. 'n Bekende gedragstoets wat die verslawingspotensiaal van 'n middel ondersoek, is die gekondisioneerde-plek-voorkeur (GPV)-toets waar die belonende of negatiewe eienskappe van 'n middel dien as 'n stimulus wat verband hou met 'n spesifieke omgewing. Hierdie omgewing word volgens Pavloviaanse beginsels later geassosieer met die spesifieke stimulus wat die middel veroorsaak. Met hierdie toets sal die verslawende of belonende middels die tyd verleng wat diere in die dwelmmiddel-gespaarde kompartement deurbring na kondisionering. Verskeie ander toetse, bv. die sukrose-voorkeur-toets (SVT) kan aandui of middels die DA-geïnnerveerde beloningsbane in die brein aktiveer deur die voorkeur vir 'n sukrose-oplossing te verhoog of te verlaag. 'n Verandering in die lokomotoriese aktiwiteit (voorwaartse beweging) van die dier word ook waargeneem na die toediening van sekere dwelmmiddels en stem dus ooreen met die psigomotoriese-stimulant-teorie dat meeste middels van misbruik die beweging van diere in 'n oop-veld-toets (OVT) verhoog. Die OVT kan ook ondersteunend wees in die bevestiging van die GPV-toetsresultate waar beweging van kardinale belang is. Die huidige studie het ten doel om die verslawingspotensiaal van efavirenz te ondersoek in beide 'n sub-akute (6 dae) en sub-chroniese (14 dae) studie. Die studie (etiese goedkeuringsnommer: NWU-00267-16-S5) het in totaal 84 manlike Sprague Dawley rotte gebruik (7 groepe met 12 rotte per groep) wat geteel en gehuisves is in die

DST/NWU PCDDP Vivarium. In die sub-akute studies is 5 groepe rotte blootgestel aan intraperitoneale inspuitings met (5, 10 of 20 mg/kg/d) efavirenz, 1mg/kg/d metamfetamien (MA) (positiewe kontrole) of 'n geneesmiddel-draagstof om die mees belonende (en dus potensieel verslawende) dosis van efavirenz vas te stel in die GPV-toets. In die sub-akute studie is MA ook met die geneesmiddel-draagstof vergelyk om sodoende die GPV- toets onder ons spesifieke laboratoriumtoestande te valideer. In die sub-chroniese studie is die GPV-toets, die SVT en die OVT met die mees belonende dosis efavirenz (5mg/kg soos vasgestel in die sub-akute studie) uitgevoer, om die effekte van die middel op die verslawende gedrag in die GPV-toets, verandering in hedonie (in die SVT) en veranderinge in voorwaartse beweging (in die OVT), te ondersoek. Weefselvlakke van DA, 5 HT en hul onderskeie metaboliëte, 3,4-dihidroksiefenielasynsuur (DOPAC) en 5-Hidroksie-indoolasynsuur (5-HIAA) asook NA is in die frontale korteks, striatum en hippokampus met behulp van hoëverrigting-vloeistofchromatografie met elektrochemiese deteksie, bepaal. Lipied peroksidase (in die brein) en superoksied dismutase (SOD) (in die plasma) is geanaliseer dmv ensiem-immunobepalings (ELISA). Die resultate van die sub-akute studie dui daarop dat 5mg/kg efavirenz die tyd wat rotte in die dwelm-gepaarde kompartement deurgebring het na kondisionering, in vergelyking met 'n geneesmiddel-draagstof kontrolegroep, verleng het. Hierdie resultate was vergelykbaar met dié van MA in dieselfde toets. Efavirenz (10 mg/kg) het geen veranderinge meegebring nie, maar 20 mg/kg het 'n beduidende vermindering veroorsaak in die tyd wat rotte in die dwelm-gepaarde kompartement deurgebring het. In die sub-chroniese studie het 5 mg/kg die tyd wat die rotte in die dwelm-gepaarde kompartement deurgebring het, verleng maar geen veranderinge in die SVT of die OVT meegebring nie. In die frontale korteks was DA en 5 HT vlakke verhoog maar 5 HIAA vlakke was beduidend verlaag. Die DA- en 5 HT-omsettempo was beduidend laer in die efavirenz groep in vergelyking met die kontrole groep. In die striatum was DA, 5 HT en NA vlakke beduidend verhoog terwyl DOPAC vlakke asook die DA-omsettempo verlaag was in die efavirenz-blootgestelde rotte. 'n Verlagings in DA, DOPAC, 5 HIAA asook 5 HT-omsettempo en 'n verhoging in die DA-omsettempo is in die hippokampus gevind. Perifere (plasma) SOD vlakke was beduidend hoër in die efavirenz groep rotte in vergelyking met die draagstof-kontrole groep, terwyl verhoogde lipiedperoksidase in die DA-ryke areas van die brein (frontale korteks en die striatum) waargeneem is. Die bevindings van die sub-akute studie dui op 'n dosis- verwante belonende effek van efavirenz met die beste resultate by die laer dosis. Hoër dosisse het negatiewe effekte in die rotte veroorsaak. Die bevindings dat sukrose-voorkeur nie in die sub-chroniese groep rotte verander het nie, ondersteun die waarneming dat die laer dosis efavirenz belonend is, aangesien geen anhedonie gedurende die tydperk van blootstelling gemanifesteer het nie. Onveranderde beweging in die OVT dui daarop dat efavirenz moontlik nie sy effekte op dieselfde wyse as ander dwelmmiddels ontlok nie en ondersteun die

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Sleutelwoorde: efavirenz, nyaope, whoonga, gekondisioneerde-plek-voorkeur, middelmisbruik. Monoamiene, oksidatiewe stress.

Congress contributions

Abstract for podium presentation at Biological Psychiatry Congress, 14 – 17 September 2017, Lord Charles Hotel, Somerset-West. (SANS Parallel Session)

Title:

Evaluation of efavirenz on addictive-like behaviours in rats: an acute and chronic treatment study

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Introduction: HIV positive patients treated with the antiretroviral drug efavirenz have been observed to experience various neuropsychiatric symptoms. Moreover, efavirenz is regularly abused by HIV positive and non-infected people by crushing and smoking this lifesaving medication in a concoction of drugs known as Nyope. This study aimed to assess the addictive-like properties of efavirenz after acute and chronic exposure in rats.

Methods: The present study (ethics: NWU - 00267- 16- S5) used a total of 84 male Sprague Dawley rats (12 per group), exposed to i.p injections of 5, 10 and 20 mg/kg efavirenz, 1 mg/kg methamphetamine (MA) (as a positive control) and vehicle for 6 days in an acute treatment paradigm using the biased study design of the conditioned place preference (CPP) test. The chronic study was conducted using the most rewarding dose of efavirenz (5 mg/kg as established in the acute study), dosed as above for 14 days. CPP, sucrose preference and locomotor activity in the open field test were assessed in the chronic study. One-way ANOVA with Dunnett's post hoc test was used for statistical analysis with a P value of 0.05 and smaller deemed significant.

Results: CPP in the acute study observed that 5 mg/kg efavirenz induced a significant increase in the time spent in the drug-paired chamber compared to the control group.

These results were comparable to the rewarding effects of MA in the same test. Efavirenz at 10 mg/kg showed no changes, while 20 mg/kg showed a significant decrease in the time spent in the drug paired chamber in comparison to the control group. The chronic study indicated a significant increase in the time spent in the drug paired chamber in comparison to the control group, although no changes in locomotion and sucrose preference were observed.

Conclusion: The findings in the acute and chronic study demonstrate a significant dose dependant rewarding effect of efavirenz in rats, with lower doses being most effective in this regard, and highlighting the abuse potential of this agent in humans. Higher doses are distinctly aversive, at least following acute treatment.

Keywords: efavirenz; conditioned place preference; drug abuse; Nyaope

Abstract for podium presentation (basic pharmacology) at South African Society for Basic and Clinical Pharmacology congress, 2-5 October 2017, University of the Free State (UFS), Bloemfontein. The student, as presenting author, won the 1st prize in the “Basic Pharmacology” category.

Title:

Evaluation of efavirenz on addictive-like behaviours and neurochemistry in rats

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Results: In the acute study it was observed that 5 mg/kg efavirenz induced a significant increase in the time spent in the drug-paired chamber compared to the control group in the CPP test. These results were comparable to the rewarding effects of MA in the same test. Efavirenz at 10 mg/kg showed no changes, while 20 mg/kg showed a significant decrease in the time spent in the drug paired chamber in comparison to the control group. Rats exposed chronically to efavirenz indicated a significant increase in the time spent in the drug paired chamber in comparison to the control group, although no changes in locomotion and sucrose preference were observed. In the chronically exposed efavirenz animals, a significant increase in cortical DA, DOPAC and 5-HT and striatal DA, 5-HT and NA was observed along with a significant decrease in cortical 5-HIAA and striatal DOPAC compared to the control group.

Conclusion: The findings in the acute and chronic study demonstrate a significant dose dependant rewarding effect of efavirenz in rats, with lower doses being most effective in this regard. The findings are in line with other studies showing that drugs of abuse increase regional brain DA, 5-HT and NA levels, driving motivational behaviour and reward, induce euphoria and arousal and cause relapse and craving. This study highlights the abuse potential of efavirenz in humans.



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**YOUNG SCIENTIST AWARD
IN BASIC PHARMACOLOGY
- PODIUM PRESENTATION**

Hereby it is certified that

JACO FOURIE

*was awarded the 1st prize
on 3 October 2017*

President

Vice -President



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List of abbreviations

A

AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ART	Anti-retroviral therapy
ARV	Antiretroviral (drug)
ATL	Analytical technology laboratory
ATP	Adenosine triphosphate

C

cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CNS	Central nervous system
CPP	Conditioned place preference

D

DA	Dopamine
DAD	Diode Array Detector
DAT	Dopamine transporter
DGL	Diacylglycerol lipase
DoH	Department of health
DOPAC	3,4-dihydroxyphenylacetic acid

E

EC	Endocannabinoid
EC-R	Endocannabinoid receptor
EDTA	Ethylene-diaminetetraaceticacid
EPM	Elevated plus maze

F

FDA	Food and drug administration
FST	Forced swimming test

G

GABA	Gamma aminobutyric acid
------	-------------------------

GSH	Glutathione
H	
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HPLC	High performance liquid chromatography
I	
i.p.	Intra peritoneally
IS	Internal standard
L	
LC	Locus coeruleus
LSD	Lysergic acid diethylamide
M	
M	Muscarinic
MA	Methamphetamine
MAO	Monoamine oxidase
MDA	Malondialdehyde
MDMA	3,4-methylenedioxymethamphetamine
MHPG	3-methoxy-4-hydroxyphenylglycol
N	
NA	Noradrenaline
NAcc	Nucleus accumbens
NAT	Noradrenaline transporter
NMDA	N-methyl-D-aspartate
NNRTI'S	Non-nucleoside reverse transcriptase inhibitors
NRTI'S	Nucleoside reverse transcriptase inhibitors
O	
OFT	Open field test
P	
PET	Positron emission tomography
PFC	Prefrontal cortex

PI	Protease inhibitors
PND	Post-natal day
R	
ROS	Reactive oxygen species
S	
SACENDU	South African Community Epidemiology Network on Drug Use
SD	Sprague-Dawley
SEM	Standard error of the mean
SERT	Serotonin transporter
SOD	Superoxide dismutase
SPT	Sucrose preference test
T	
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
Δ^9 -THC	Delta-9-tetrahydrocannabinol
V	
VMAT-2	Vesicular monoamine transporter-2
VTA	Ventral tegmental area
W	
WST	Water-soluble tetrazolium
X	
XO	Xanthine oxidase
Numbers	
2-AG	2-Arachidonoylglycerol
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin

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Chapter 1

Introduction

This chapter serves as an introductory chapter describing the study as a whole. In this regard, the approach and layout of the dissertation, the problem statement with a short literature review (to be elaborated on in Chapter 2), study questions, the project aims, hypothesis, project layout and expected results will be discussed.

1.1 Dissertation layout

This dissertation is presented in the article format where key data is presented as a manuscript aimed for publication in a specific journal (Chapter 3). Key elements of the study such as Methods, Results and Discussion, are therefore presented in the article, i.e. Chapter 3. The addenda contain additional methods, materials, results and discussion and are presented at the end of the dissertation. For assistance in finding specific elements in the study, the following outline can be used:

- Chapter 1
 - Problem statement, objectives and project layout
- Chapter 2
 - Literature review
- Chapter 3
 - Article
 - literature review
 - materials and methods
 - results and discussion
- Chapter 4 (general discussion of findings)
 - General discussion
- Chapter 5
 - Summary and conclusion
- Addendum A
 - Validation of the conditioned place preference (CPP) paradigm
- Addendum B
 - Neurochemical and peripheral analysis

- Addendum C
 - Authors instructions for *Oxidative Medicine and Cellular Longevity*.

1.2 Problem statement

Based on data from 2014, acquired immunodeficiency syndrome (AIDS) related deaths in eastern and southern Africa peaked at around 1,1 million (UNAIDS, 2015). A 62% reversal of these numbers was observed in 2016, with 1 600 000 new infections having been successfully averted due to antiretroviral therapy (ART) (UNAIDS, 2016). More than 56% of people living with human immunodeficiency virus (HIV) receive ART which consists of two nucleoside reverse transcriptase inhibitors (NRTI's) such as zidovudine and lamivudine and a drug from either one of the following three classes, the protease inhibitors (PI) (such as ritonavir), integrase strain inhibitors (such as raltegravir) or a non-nucleoside reverse transcriptase inhibitors (NNRTI) such as efavirenz (Staszewski *et al.*, 1999; Meintjes *et al.*, 2017).

Efavirenz is one of the most popular NNRTI's (Staszewski *et al.*, 1999) for the treatment of HIV-type1 (HIV-1) infection due to its superior virological efficacy (Arribas, 2003; Best & Goicoechea, 2008; Sierra-Madero *et al.*, 2010). However, after administration of efavirenz, up to 50% of patients receiving treatment experience central nervous system (CNS) side-effects which include insomnia, dizziness, somnolence, vivid and abnormal dreams and thoughts, difficulty concentrating, suicidal ideation and manic episodes (Staszewski *et al.*, 1999; Marzolini *et al.*, 2001; Kryst *et al.*, 2015). A previous study also indicated that hallucinations are among the most common side-effects of efavirenz use (Lochet *et al.*, 2003). This psychedelic-like side-effect profile of efavirenz clearly shows that the drug causes CNS modulation which could be linked to possible abuse and dependency. In line with this hypothesis, news reports in South Africa started surfacing from as early as 2010, reporting people crushing and smoking efavirenz in a combination with marijuana as well as various other constituents in a mixture commonly known as "Nyaope" or "Whoonga" (Hull, 2010; Cullinan, 2011; Fihlani, 2011; Rough *et al.*, 2014).

Such abuse of this HIV drug could possibly reduce the supply to patients receiving treatment and place tremendous pressure on the Department of Health (DoH) with respect to its antiretroviral role out plan. Moreover, the lives of patients receiving treatment with this drug have become endangered due to an increase in criminal activity (Cullinan, 2011). Indeed, various studies have reported people being mugged or robbed of their medicines upon returning from their consultations (Cullinan, 2011; Rough *et al.*, 2014). According to Rough *et al.* (2014), participants in a study acknowledged that not all ARV's were being stolen, with efavirenz identified as the drug of choice being stolen, sold and used recreationally.

Chapter 1: Introduction

To highlight the problem even more, people being exposed to regular recreational use of ARV drugs like efavirenz are at high risk of acquiring pre-treatment resistance to NNRTI's, with mutations that confer resistance to such agents being the main driver of this phenomenon. Efavirenz-related resistance is among the most common in this regard (Langmann *et al.*, 2002; Ngaimisi *et al.*, 2010).

Very little scientific proof of the abuse of this drug exists, with only one article stating that the neuroreceptor binding properties and psychoactive effects of efavirenz is in line with that of lysergic acid diethylamide (LSD), a widely abused psychedelic drug (Gatch *et al.*, 2013). Of great concern is that a new drug, dolutegravir, a novel integrase strain inhibitor, was recently approved by the Food and Drug Administration (FDA) for the treatment of HIV-1 and is now at advanced stages of clinical development (Eron *et al.*, 2010). Dolutegravir displays activity against most strains of HIV-1 resistant to raltegravir, but has also been highlighted to have similar neuropsychiatric side-effects as efavirenz (Yagura *et al.*, 2017). Consequently, the addictive-like phenomenon of these important medicines poses a potential risk on health, making it imperative that research is undertaken to uncover the underlying mechanisms of how efavirenz targets the neurocircuitry of reward, its possible addiction biomarkers and how this relates to its abuse potential.

Moreover, when studying drugs of abuse, it is important to know that various factors such as genetics and environmental factors play significant roles in addiction development (Goldstein & Volkow, 2002). However preclinical studies on drugs of abuse show that there are common biological pathways and changes mediating the effects of these drugs (Goodman, 2008). It is well established that most drugs of abuse involve pathways of reward that are under the direct influence of the dopamine (DA)-ergic system (Goldstein & Volkow, 2002). Serotonin (5-HT) and noradrenaline (NA) also play crucial roles in mediating the rewarding and addictive-like effects of these drugs, with pre-clinical studies on drugs of abuse indicating alterations in these monoamine levels in the prefrontal cortex (PFC), striatum and hippocampus after administration of illicit drugs (Gibbs & Summers, 2002; Müller *et al.*, 2007). Moreover, studies have observed neurotoxicity in DA rich brain regions after the use of drugs of abuse such as MA (reviewed in Cunha-Oliveira *et al.* (2008)). In this regard, the metabolites of DA such as 3,4-dihydroxyphenylacetic acid (DOPAC), as well as reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and various quinone derivatives of DA, lead to lipid peroxidation and neuronal damage if the ensuing oxidative stress exceeds the capacity of the body's natural anti-oxidant systems (eg. superoxide dismutase, or SOD) (Cunha-Oliveira *et al.*, 2008). This neurotoxicity induced by drugs of abuse may ultimately induce not only addictive behaviours, but also numerous other behavioural alterations, such as anxiety,

changes in locomotion, psychosis and deficits in learning and cognition (Harkany *et al.*, 1999; Müller *et al.*, 2007; Amos-Kroohs *et al.*, 2015).

Various preclinical behavioural tests can be used to screen a specific drug for its rewarding or addictive-like properties, with the most popular being the CPP test (Bardo & Bevins, 2000). This test assesses the rewarding or aversive effects of a drug through means of classical conditioning (Bardo & Bevins, 2000; Tonissaar *et al.*, 2006). Classical conditioning refers to the learning process of continuously pairing a stimulus (in this case a rewarding drug) with a previously neutral stimulus (the drug-paired compartment) leading to a conditioned stimulus (Robinson & Berridge, 2013). This stimulus can become an incentive stimulus, making it motivationally attractive (Berridge & Robinson, 1998; Robinson & Berridge, 2013). Methamphetamine (MA) has continuously shown positive rewarding and reinforcing effects in the CPP test (Zakharova *et al.*, 2009) which makes this drug a valuable positive control to validate the CPP paradigm. Another preclinical behavioural test that can be used to evaluate the possible stimulatory effects of drugs of abuse is the locomotor activity test (performed in an open field test (OFT) arena), with previous studies indicating that drugs of abuse such as opiates and stimulants induce an increase in locomotor activity (Villégier *et al.*, 2003; Weinshenker & Schroeder, 2007; Aarde *et al.*, 2013). Although not strictly a test to measure addictive-like behaviour, the sucrose preference test (SPT) used in preclinical studies, measures hedonic activity, wherein an animal actively seeks (or avoids) a pleasurable experience (Der-Avakian & Markou, 2012). The SPT is used to indicate whether a drug of abuse increases the intake of sucrose through activation of the reward pathways in the brain (Der-Avakian & Markou, 2012).

Thus, identifying neurochemical and oxidative stress related markers as well as specific behavioural alterations may be valuable in the investigation of possible drugs of abuse and would also apply to this study on efavirenz.

1.3 Study questions

A number of study questions arise in the evaluation of the above-mentioned problem statement:

- 1) Can a CPP paradigm be validated in our laboratory to assess the possible rewarding properties of efavirenz, as compared to a positive control, MA, and produce results similar to previously published studies in this paradigm?
- 2) Will sub-acute efavirenz induce addictive-like behaviour in the above-mentioned paradigm, and how will it compare to the positive control?

- 3) Will addictive-like behaviour in the CPP test (if any) induced by sub-acute efavirenz be dose specific?
- 4) Will sub-chronic efavirenz (at a dosage established in the sub-acute study) also produce addictive-like behaviour in the CPP test?
- 5) Will sub-chronic efavirenz exposure lead to changes in locomotor activity and hedonic-like behaviour as measured by assessing preference for sucrose, in line with known drugs of abuse?
- 6) Will sub-chronic efavirenz induce any regional brain monoamine alterations and lipid peroxidation alterations?
- 7) Will sub-chronic efavirenz induce any alterations in central (lipid peroxidation) or peripheral (SOD) markers of oxidative stress?

1.4 Project aims

To answer the above-mentioned questions, the following aims were proposed:

- 1) To validate the CPP paradigm in our laboratory by using MA as a positive control at a dose of 1mg/kg, which has been shown to produce a definite preference for the drug-paired compartment after conditioning in rats due to superior rewarding properties established in previous studies (Masukawa *et al.*, 1993; Zakharova *et al.*, 2009).
- 2) Evaluate the possible addictive-like behaviour (assessed in the CPP test) induced by sub-acute efavirenz exposure at three different dosages (viz. 5, 10, 20 mg/kg/day), in order to determine the most rewarding dosage of efavirenz in rats, and compare these to MA.
- 3) To investigate the effect of sub-chronic efavirenz administration (at the most rewarding dose as determined in the sub-acute study) on locomotor activity (in the OFT), preference for sucrose (in the SPT) and addictive-like behaviour (in the CPP) in rats, compared to rats only receiving vehicle. MA (1 mg/kg) will not be used in the sub-chronic study.
- 4) To investigate the effect of sub-chronic efavirenz administration (at the most rewarding dose determined in the sub-acute study) on regional brain monoamines (viz. DA, 5-HT, NA and related metabolite levels), regional brain lipid peroxidation, as well

as plasma levels of SOD in rats, compared to rats only receiving vehicle. MA (1 mg/kg) will not be used in the sub-chronic study

1.5 Hypothesis

We hypothesize that sub-acute MA administration in rats will induce addictive-like behaviour in the CPP test, with rats exposed to sub-acute efavirenz similarly showing addictive-like behaviour in the CPP, but in a dose-dependent manner compared to a vehicle control group. Further, sub-acute efavirenz (at a specific dosage) administration in rats will induce similar addictive-like behaviour in the CPP test comparable to rats exposed to MA, seeing that MA is a reliable positive control. We also propose that sub-chronic administration of efavirenz (at the most rewarding dosage established in the sub-acute study) in rats will induce various behavioural alterations representative of a drug of abuse, as assessed in the OFT, CPP and SPT, together with distinct regional brain monoamine alterations as well as evidence for oxidative stress in both peripheral and central compartments, compared to a vehicle control group.

1.6 Project layout

This study consists of two cohorts (see Figure 1-1), viz: A, a sub-acute study (alternate day dosing over 6 days) validating the CPP where rats are exposed to MA (1 mg/kg) administration (Zakharova *et al.*, 2009) as positive control (compared to vehicle alone), and three different dosages of efavirenz (5-20mg/kg), the latter in order to investigate a dose-response relationship under the same conditions (Figure 1-1A); and B, a sub-chronic study (alternate day dosing over 14 days) where rats exposed to the most rewarding sub-acute dose of efavirenz are compared to a vehicle control group (Figure 1-1B) with respect to specific behavioral, neurochemical and peripheral parameters.

In the sub-acute study, the most rewarding dosage of efavirenz was determined by comparing three different dosages, viz. 5, 10, 20 mg/kg, to a vehicle control. Each of the above-mentioned exposure groups consisted of 12 male Sprague Dawley (SD) rats. A total of 84 animals were used, weighing between 250 and 300 grams at the start of treatment. Drug exposure lasted for 6 days in the sub-acute study and for 14 days in the sub-chronic study (Figure 1-1 A and B respectively). The following behavioral analyses were conducted in the sub-chronic study: the CPP, SPT and the OFT. All animals were euthanized 24hrs after the last behavioral test (which was the SPT) and regional brain tissue and trunk blood collected for peripheral and neurochemical analysis, as noted earlier.

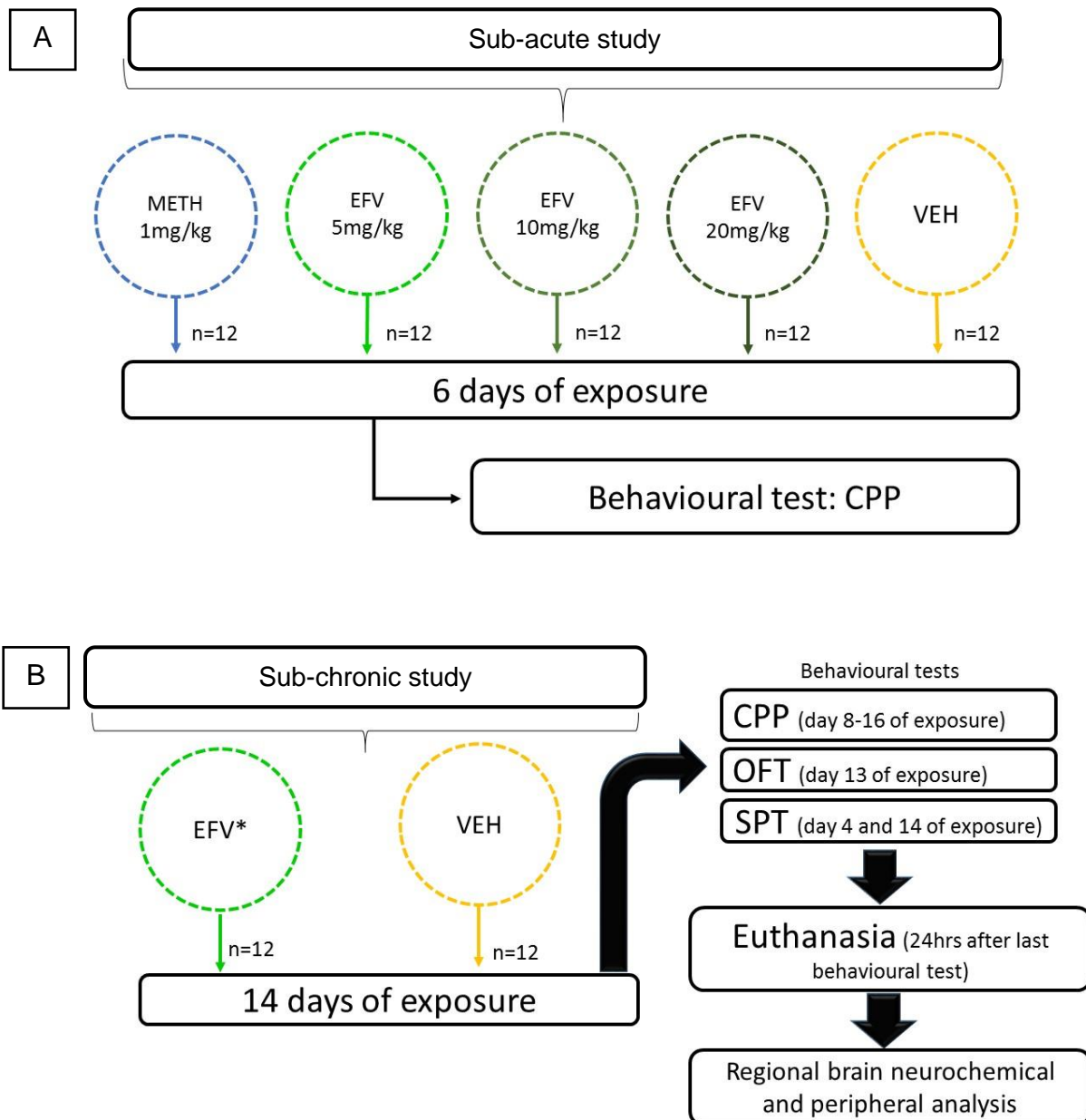


Figure 1-1: Study design of (A) the sub-acute and (B) the sub-chronic studies. A) Rats (n=12 per group) will be exposed to alternate day dosing of methamphetamine (*METH*) at 1 mg/kg, efavirenz (*EFV*) at either 5, 10 or 20 mg/kg or a vehicle (*VEH*) for 6 consecutive days during which the conditioned place preference test (*CPP*) will be performed. B) Rats will be exposed to the most rewarding dosage (*EFV**) (as determined in the sub-acute study) or a vehicle for 14 alternating days during which the *CPP* test, open field test (*OFT*) and the sucrose preference test (*SPT*) will be done. Regional neurochemical analysis will be done in the frontal cortex, striatum and hippocampus and peripheral analysis in the plasma.

1.7 Expected results

We proposed the following outcomes:

- Rats exposed to sub-acute MA (1mg/kg) will have a significant preference for the drug-paired chamber after conditioning, compared to rats only receiving vehicle, thereby validating the CPP under our laboratory conditions.
- When investigating the addictive-like behaviour (in the CPP) in rats exposed to sub-acute dosages of efavirenz (5, 10 and 20 mg/kg), we expect a distinct dose-response relationship (with higher doses being most rewarding) to be evident across the three dosages vs. rats receiving only vehicle.
- When comparing efavirenz to the positive control in the sub-acute study, viz. MA, we predict that rats exposed to either one of the three dosages of efavirenz will show a preference for the drug-paired compartment comparable to that produced by MA.
- Rats exposed to sub-chronic efavirenz (dosage established in the sub-acute study) will have a significant preference for the drug-paired compartment in the CPP test, compared to the vehicle control group.
- Rats exposed to sub-chronic efavirenz (dosage established in the sub-acute study) will show increased locomotor activity in the OFT, compared to the vehicle control group.
- Rats exposed to sub-chronic efavirenz (dosage established in the sub-acute study) will show increased preference for sucrose in the SPT, compared to the vehicle control group.
- Rats exposed to sub-chronic efavirenz (dosage established in the sub-acute study), will also have significant regional brain neurochemical (DA, 5-HT, NA and related metabolite levels) alterations compared to the vehicle control group, in line with previous studies evaluating MA and other drugs of abuse, such as:
 - i. Significant increases in DA levels in the frontal cortex, striatum and hippocampus.
 - ii. Increased 5-HT levels in the frontal cortex and the striatum.
 - iii. Increased levels of NA will be observed on all three the brain regions in
 - iv. Increased DOPAC and 5-HIAA levels in the frontal cortex and the striatum and hippocampus.
- Higher levels of regional brain lipid peroxidation and peripheral oxidative stress (plasma SOD) will be observed in rats exposed to sub-chronic efavirenz compared to the control group.

1.8 Ethical consideration

Animals 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 NWU. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the NWU. Animals were maintained and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation (Ethics approval number NWU-00267-16-A5).

This study will shed light on the recently reported concerns for the recreational use of efavirenz by examining its effects on addictive-like behaviours in rats and concomitant effects on neurobiological markers related to traditional drugs of abuse.

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Chapter 2

Literature review

2.1 Human Immunodeficiency Virus (HIV) and antiretroviral therapy (ART)

In 2014 the number of acquired immunodeficiency syndrome (AIDS) related deaths peaked at around 1.1 million in eastern and southern Africa (UNAIDS, 2015). However, due to antiretroviral therapy (ART), a decline of almost 62% was observed in the year 2016 (UNAIDS, 2016). More than 56% of people with human immunodeficiency virus (HIV) are currently on ART, consisting of six drug categories viz. (1) nucleoside reverse transcriptase inhibitors (NRTI's), (2) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (3) entry inhibitors, (4) fusion inhibitors, (5) integrase inhibitors, and (6) protease inhibitors (PI's) (Ramana *et al.*, 2014). First line ART consists primarily of two NRTI's such as zidovudine and lamivudine and one NNRTI such as efavirenz ((4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one) (Meintjes *et al.*, 2017). The NNRTI's were first introduced in 1994 together with the PI's which, when used in combination, transformed this previously fatal disease into one that could be controlled indeterminately (Treisman & Soudry, 2016). The NNRTI's mediate their effect by non-competitively inhibiting reverse transcriptase and are therefore very effective inhibitors of HIV replication (Wise *et al.*, 2002). The first NNRTI to be approved was niverapine. However, this drug is considered inferior to efavirenz, which is the drug of choice in most prescribing guidelines due to its superiority with respect to virological suppression (Wise *et al.*, 2002).

2.1.1 Efavirenz

Efavirenz is a highly lipophilic drug (Lamorde *et al.*, 2012), able to penetrate the central nervous system (CNS) and the spinal fluid and thereby effectively inhibit strains of multi-drug resistant proteins in the CNS (Adjene & Igbigbi, 2015). The neuropsychiatric effects of efavirenz have long been debated until recently when such evidence became more substantial and therefore warranted more attention (Apostolova *et al.*, 2015). After administration of efavirenz, up to 50% of patient's receiving treatment experience a profile of CNS side-effects (Kryst *et al.*, 2015), such as insomnia and sleep alterations, fatigue, dizziness, vertigo, confusion, somnolence, vivid and abnormal dreams and thoughts, difficulty concentrating, suicidal ideation, manic episodes, nervousness, irritability and even in some cases euphoria (Staszewski *et al.*, 1999; Marzolini *et al.*, 2001; Fumaz *et al.*, 2002; Kryst *et al.*, 2015). Kenedi

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and Goforth (2011) also warned that efavirenz may induce the expression of psychotic and manic episodes after administration to at-risk drug addiction populations. Furthermore, more worrying side-effects of efavirenz include depression, anhedonia and cognitive impairment (Blanch *et al.*, 2001; Fumaz *et al.*, 2002; Fumaz *et al.*, 2005; O'Mahony *et al.*, 2005). Another cause for concern is efavirenz-induced neurotoxicity due to possible interference with energy metabolism by diminishing cellular adenosine triphosphate (ATP) levels (Brown *et al.*, 2014). A recent preclinical study found that efavirenz not only increases oxidative stress proteins, but also endoplasmic reticulum stress and autophagy in human endothelial cells, which are clear indicators of cell stress and cell damage (Weiß *et al.*, 2016). Efavirenz is mainly metabolized by the cytochrome P450 enzymes, specifically subtype 2B6, to the primary metabolite 8-hydroxy-efavirenz (Brandmann *et al.*, 2013). This metabolite is toxic in neuronal cultures, with the neurotoxicity being directly related to the concentration of efavirenz in the plasma (Declodt & Maartens, 2013). Its primary mechanism of toxicity is reported to be via the induction of neuronal damage through inhibiting mitochondrial respiration (Tovar-y-Romo *et al.*, 2012; Brandmann *et al.*, 2013). Metabolism of efavirenz to 8-hydroxy-efavirenz is highly dependent on the metabolic pathway discussed above and genetic factors that influence its metabolism (*viz.* slow or rapid metabolisers) that will eventually alter the concentrations of this metabolite (Brandmann *et al.*, 2013). Furthermore, this metabolite is thought to be implicated in the CNS adverse events observed in efavirenz treated patients (Apostolova *et al.*, 2017) with one study observing potent neurotoxic effects which may ultimately lead to cell damage (Tovar-y-Romo *et al.*, 2012). Although the mechanism through which efavirenz elicits its effects are still unclear, efavirenz has been observed to interact with the serotonin (5-HT)_{2A} receptor subtype which leads to lysergic acid diethylamide (LSD)-like effects in rats (Gatch *et al.*, 2013). However, another pre-clinical study ascribed hallucinations and cognitive impairment to the ability of efavirenz to completely or partially block agonist binding at muscarinic (M)₁ and M₃ receptors, thus equating efavirenz to the M-antagonists scopolamine and atropine which are widely known to induce hallucinations and memory impairment (Dalwadi *et al.*, 2016; Cavalcante *et al.*, 2017). Further studies also suggest that CNS-related adverse events associated with efavirenz are directly related to drug concentration (Marzolini *et al.*, 2001).

This psychedelic side-effect profile of efavirenz clearly shows that the drug causes CNS modulation that could be linked to possible abuse and dependency. In line with this hypothesis are recent news reports in South Africa describing people crushing and smoking efavirenz, and sold on the black market (Rough *et al.*, 2014) in combination with various constituents including marijuana; this mixture is commonly now as “Nyaope” or “Whoonga” (Hull, 2010; Cullinan, 2011; Fihlani, 2011; Rough *et al.*, 2014).

Dolutegravir, a novel integrase strand inhibitor, has recently been approved by the Food and Drug Administration (FDA) for the treatment of HIV-1 and is in advanced clinical testing (Eron *et al.*, 2010) against HIV. It displays activity against most strains of HIV-1 resistant to raltegravir, but presents with similar neuropsychiatric side-effects as efavirenz (Yagura *et al.*, 2017). The addictive-like phenomenon of these medicines is noteworthy and poses a potential global risk on health in a manner only hitherto experienced with efavirenz.

2.2 Drug abuse and addiction

In South Africa, surveys pertaining to substance abuse are not regularly performed and therefore it is difficult to estimate trends and changes over time (Pasche & Myers, 2012). However, a few recent surveys have included questions on substance abuse of which the most common are the Youth Risk Behaviour Survey, South African Demographic and Health Survey and the National HIV Survey (Reddy *et al.*, 2003; Pasche & Myers, 2012). These surveys aim to shed light on substance abuse but are not carried out on a regular basis and so are unable to track the changes in abuse over a longer time period. The South African Community Epidemiology Network on Drug Use (SACENDU) collects data on substance abuse by making use of the total admissions for substance abuse related matters, with data from as early as 1996 being collected (Pasche & Myers, 2012). Such data suggests that alcohol abuse is the most prevalent, with 28-39% of the population abusing alcohol, while cannabis and amphetamines suggest a prevalence of 2% and 0,2% respectively (Shisana, 2005). Because these studies have so many shortcomings, the prevalence of substance abuse is extremely underestimated. In fact, according to the South African Youth Risk Behaviour Survey of 2008, cannabis use among the youth is as high as 13%, while in certain provinces in South Africa the use of heroin and methamphetamine (MA) is greatly underestimated (Reddy *et al.*, 2003; Shisana, 2005). Very little proof of the degree of the recreational use of efavirenz exist and the abuse of efavirenz may be greatly underestimated.

The number of studies and theories on drug abuse and addiction are overwhelming and it is therefore important to first distinguish between the terms “drug abuse” and “drug addiction” (alternatively called dependence). Drug abuse is defined as the use of a drug that is not in accordance with the approved manner of use or the recurrent use of illegal drugs (Koob, 2000; Leonard, 2004; Koob, 2006). Drug abuse does not describe a particular pattern of abuse but rather a pattern of drug use which is not acceptable in a particular culture, and covers the use of licit as well as illicit drugs for their pleasurable effects, such as the use of amphetamine, over the counter drugs and alcohol (Leonard, 2004). Drug dependence on the other hand is seen as a complex disease of the brain as result of constant intake or intoxication with a specific drug, resulting in a chronically relapsing disorder recognized by drug seeking and an

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overwhelming intake of a drug despite significant harmful consequences (Koob & Le Moal, 1997; Goldstein & Volkow, 2002; Goodman, 2008). In most cases of drug dependence, the dose must be increased over time to maintain the initial effects of the drug (Koob 2006). With these drugs of abuse, psychological and physical dependence occur and the lifestyle of an addictive patient is dominated by the urge and need to insure supplies of the drug (Leonard, 2004; Koob, 2006).

Drug dependence is characterised by three main factors: tolerance, and both physical and psychological dependence (Leonard, 2004). Tolerance occurs when a continuously increasing dose of the drug has to be taken to induce the same pharmacological effects in the user and can occur as a result of increased metabolism of the abused drug due to induction of the hepatic enzymes responsible for metabolizing the drug (Leonard, 2004). Physical dependence is induced by most of the drugs of abuse (whether it be opioids, sedatives, alcohol, cannabinoids or stimulants) and is characterised by abnormal behaviour and a profile of autonomic symptoms which can include increased heart rate and blood pressure, tremors, confusion, visual hallucinations and seizures after the abrupt cessation of the drug (Leonard, 2004; Weinberger *et al.*, 2010). Psychological dependence on the other hand is instigated by drugs of abuse through their euphoric effects on the user; the discontinuation of these drugs leaves the user with feelings of dysphoria and an exceptional craving for the drug and the remembered hedonic effects (Leonard, 2004; Koob, 2006). Many factors such as genetics and environmental factors play an important role in modulating drug addiction (Goldstein & Volkow, 2002) that not only impacts on its neurobiology, but also how it is treated.

One of the most popular theories of addiction is the incentive sensitisation theory (Koob, 2006; Robinson & Berridge, 2013). According to this theory, craving for drugs are governed by a sensitized neural system that consist of the brain reward system (Robinson & Berridge, 2013) (further discussed under section 2.3). In evaluating the incentive for craving, certain aspects of an environment can become cues after continuously being paired with the drug, meaning that certain stimuli in the environment associated with a drug can trigger motivation to seek the drug through Pavlovian conditioning principles (Everitt, 1997). This process describes an unconditioned stimulus that can become a conditioned stimulus after continuously being paired with the drug, leading to classical conditioned responses in the drug abuser (Everitt, 1997; O'Brien *et al.*, 1998; Robinson & Berridge, 2013). According to Robinson and Berridge (2013), normal stimuli, such as cues associated with rewards are turned into incentive stimuli, making them motivationally attractive and able to trigger an urge or craving if the substance (in this case the drug of abuse) is not taken (much like smoking). The strong association made between the stimuli and the reward ultimately leads to an intense "wanting" of the drug, called

an incentive salience (Robinson & Berridge, 2013). This pathological incentive motivation for the drug can last for years even after drug use has been discontinued (Everitt, 1997). In summary, the incentive sensitisation theory starts with the individual developing a hyper-sensitization towards a substance after continuously using or being exposed to the drug, whereupon the drug stimulates the already sensitized reward pathways leading to an initial increase in pleasure and euphoria and the induction of an incentive salience (intense wanting or craving) that drives repetitive behaviour to satiate the craving (See Figure 2-1) (Everitt, 1997; Robinson & Berridge, 2013).

Taking the above-mentioned factors into account, the focus of addiction studies has started to shift from the rewarding properties of a drug towards the motivational aspects of addictive-like behaviour (Wise, 2004b).

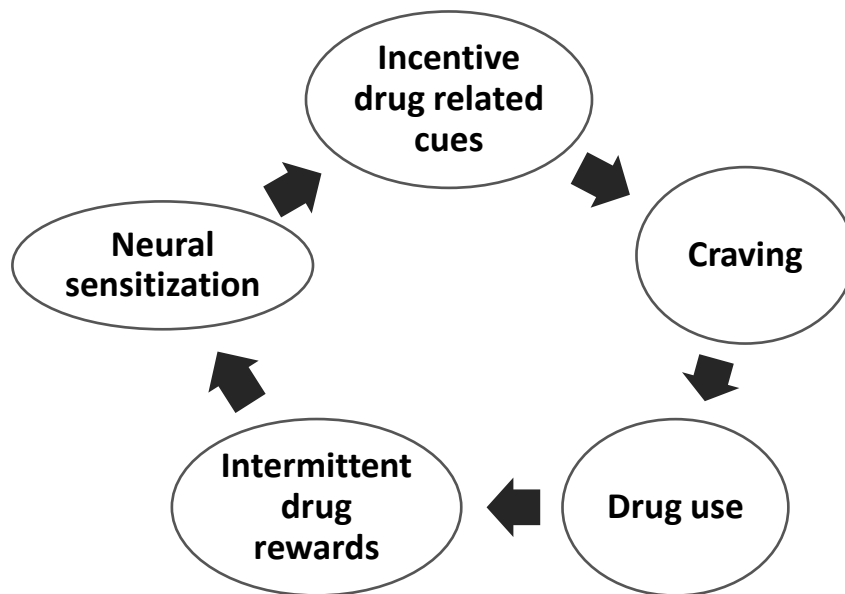


Figure 2-1: The cycle of events in the sensitization theory. Development of a hyper-sensitized neuronal system toward drug use occurs after continuous exposure to a drug, ultimately leading to drug cues inciting an intense drug craving and drug-use (Everitt, 1997; O'Brien *et al.*, 1998; Wise, 2004b; Robinson & Berridge, 2013)

2.3 Neurochemistry of addiction

2.3.1 Dopamine

Goodman (2008) hypothesised that all addictive disorders have one or more specific pattern of neurobiological activity, developmental process and brain structure in common, this despite the neurochemistry accompanying addiction not being fully understood. In fact, it is now well-

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established that the majority of drugs of abuse, and addiction as a mental disorder, involve reward processes under the control of the mesocorticolimbic system (see Figure 2-2).

In this system dopamine (DA)-ergic signalling and DA receptors (D₁, D₂, D₃) are largely responsible for reinforcement learning (Volkow *et al.*, 2003; Volkow *et al.*, 2004). The mesolimbic pathway, which includes the nucleus accumbens (NAcc), amygdala, and hippocampus, is important in drug reward, in particular reinforcement learning and memories regarding drug-use and the environment as well as conditioned and unconditioned stimuli (Goldstein & Volkow, 2002). Not only does intra-synaptic DA levels increase after administration of drugs of abuse, but these heightened levels are also observed in patients during gambling, eating and sexual behaviour, indicating that DA plays a profound role in rewarding and pleasurable experiences, i.e. hedonic activity (Goodman, 2008). Various studies show the involvement of DA in classical conditioning, indicating that the effects of DA go beyond its rewarding role (Duvauchelle *et al.*, 2000; Salamone *et al.*, 2005). Moreover, Duvauchelle *et al.* (2000) showed that classically conditioned drug stimuli are able to increase DA release in the mesolimbic region of the brain.

Recent research has also shed light on the role of DA in driving motivational behaviour of certain events (including reward) in addictive-like behaviour:

- The DAergic neurons are located in the ventral tegmental area (VTA) of the midbrain which then project to key brain regions involved in reward, as follows (refer to Figure 2-2):
 - the NAcc (responsible for motivation and drug reinforcement)
 - the amygdala (responsible for association of reward with drug-associated cues and negative reinforcement)
 - the ventral pallidum, hippocampus, and the limbic system (associated with memory and learning),
 - the forebrain, particularly the frontal cortex (associated with cognitive and social behaviour learning) (Kalivas & McFarland, 2003; Willuhn *et al.*, 2010).
- The NAcc projects mainly through inhibitory gamma-aminobutyric acid (GABA)-ergic projections directly to the VTA/substantia nigra which in turn sends GABAergic signalling to the thalamus; from here glutamatergic projections to the frontal cortex close the loop (Pierce & Kumaresan, 2006). Glutamate binds to N-methyl-d-aspartate (NMDA) receptors on the inhibitory GABA interneuron which synapses on secondary glutamate neurons in the VTA that are responsible for the release of monoamines in subcortical brain regions (Möller *et al.*, 2015). Evidence indicates that glutamate

receptors play a crucial role in modulating the behaviour and the actions of drug abuse and addiction (Kenny & Markou, 2004).

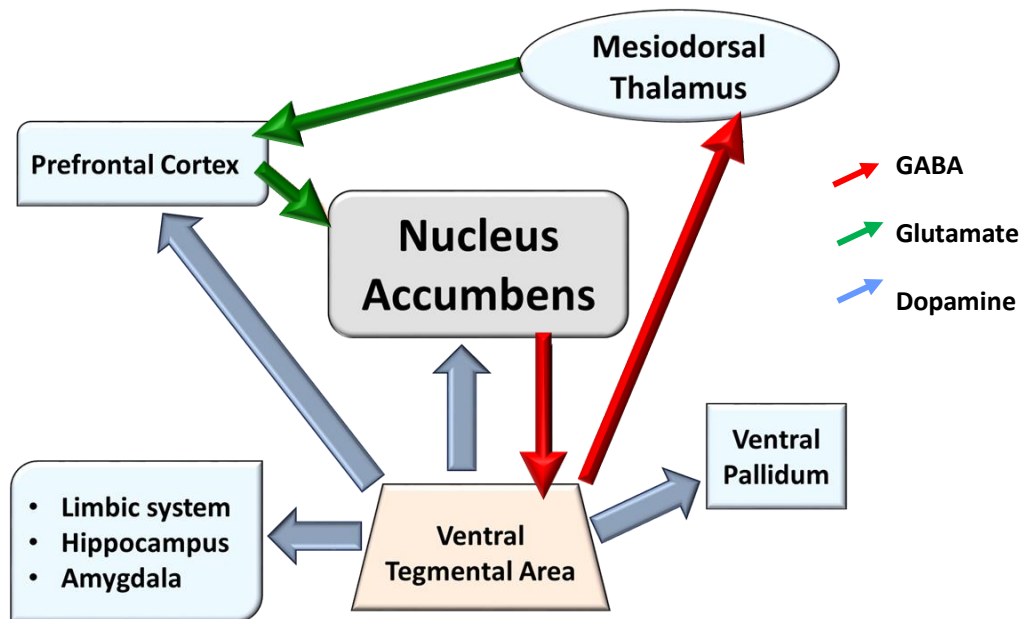


Figure 2-2: The mesocorticolimbic circuitry. Green arrows (activating) = glutamatergic pathways; red arrows (inhibitory) = GABAergic pathways; blue arrows = DAergic pathways. See text for details (adapted from Pierce and Kumaresan (2006))

Clinical and pre-clinical studies have shown that the primary motivational role of DA lies with DA projections in the NAcc and the frontal cortex (Missale *et al.*, 1998; Robinson & Berridge, 2013). It is however important to understand that DA transmission happens in either a tonic or phasic manner (Dreyer *et al.*, 2010). Tonic DA transmission refers to the low frequency firing of DA neurons, having an effect on high affinity D_2 receptors in the NAcc, whereas phasic transmission refers to the high frequency bursts of DA neurons in the NAcc after a subject is presented with a motivational stimulus predicting drug availability, leading to high concentrations of DA in the NAcc available to bind to the low affinity D_1 receptor (see Figure 2-3) (Dreyer *et al.*, 2010). Five different DA receptor subtypes are evident which are divided into two groups, viz. D_1 and D_5 , and D_2 - D_4 (see Figure 2-3) (Centonze *et al.*, 2003). The former are situated post-synaptically where they act to stimulate the adenylate cyclase-cyclic adenosine monophosphate (cAMP) cascade while the latter are mostly situated pre-synaptically (but also occur post-synaptically) and inhibit this signalling cascade (Centonze *et al.*, 2003).

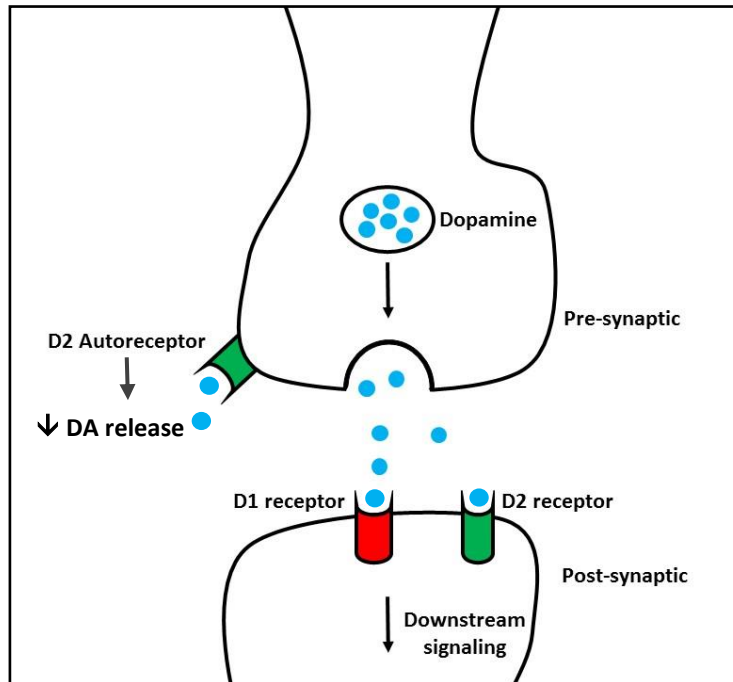


Figure 2-3: Illustration of D1 and 2 receptor location and DA binding (see text for detail). (Centonze *et al.*, 2003).

Both D₁ and D₂ receptors play a role in reward and learning behaviour associated with drugs of abuse (Missale *et al.*, 1998). D₁ receptors are abundant in the brain with the highest levels expressed in the nigrostriatal, mesolimbic, and mesocortical areas, including the striatum, NAcc, substantia nigra, amygdala and the frontal cortex (Beaulieu & Gainetdinov, 2011), while lower levels of D₁ receptor expression are observed in the hippocampus (Beaulieu & Gainetdinov, 2011). Pre-clinical studies have established that the D₁ receptor is associated with the rewarding nature of food, drugs and alcohol as well as the reinforcement of drug seeking behaviour, place preference for drugs of abuse after conditioning and enhancement of palatability of food (Karasinska *et al.*, 2005; Cooper & Al-Naser, 2006; Graham *et al.*, 2007). Furthermore, activation of the D₁ receptor alone has little effect on specific behaviours in a pre-clinical setting (discussed later) but does exert a synergistic interaction with D₂ receptors to maximise the effects on for e.g. locomotor activity (discussed under section 2.6.2) and other behaviours associated with reward (Gershanik *et al.*, 1983). Preclinical studies show that after administration of D₁ receptor antagonists, these receptors become supersensitive to DA (Haney *et al.*, 2001). Cocaine self-administration and the subjective effects of cocaine were found to be increased after administering a DA antagonist, indicating that D₁ receptor supersensitivity plays a role in the addiction process (Haney *et al.*, 2001).

The D₂ receptor on the other hand are mostly expressed in the NAcc, striatum, VTA, hypothalamus, amygdala and the hippocampus (Seeman, 2006), and present with a high

affinity for DA. After the activation of presynaptic D₂ receptors (functioning as auto receptors), DA signalling is decreased, whereas the activation of the postsynaptic D₂ receptors has the opposite effect (Missale *et al.*, 1998) (indicated in Figure 2-3). The D₂ auto-receptor can become activated at lower concentrations of DA that is needed to activate the postsynaptic receptors and can thus lead to the dual effects of DA agonists on these receptors (Missale *et al.*, 1998). Preclinical studies have shown a complex range of results when either an agonist or antagonist on the D₂ receptor was administered; D₂ agonists (e.g. quinpirole) eliminate the preference for palatable food and block the addictive-like effects of cocaine whilst the administration of a D₂ antagonist reduces the self-administration of ethanol and inhibits cocaine seeking behaviour (Graham *et al.*, 2007). Moreover, clinical studies indicate a direct correlation between addiction and lower densities of D₂ receptors in the ventral striatum (Matsumoto *et al.*, 2001). This is true for abusers of alcohol, cocaine, methamphetamine and heroine (Martin-Soelch *et al.*, 2001; Volkow *et al.*, 2003; Volkow *et al.*, 2004). In a former clinical study, non-addicted subjects were given low doses of ethanol; by utilising a positron emission tomography (PET) scan, researchers found that persons with a higher density of D₂ receptors described more pronounced feelings of being intoxicated whereas higher doses were needed to elicit an analogous response in those presenting with low D₂ receptor density (Yoder *et al.*, 2005). Furthermore, all psychostimulant drugs interact with the DAergic system, although some of their effects (such as increased locomotor activity) do not correlate with selectively blocking the DA-transporter (DAT) (Müller *et al.*, 2007). Furthermore, the role of the DAT (responsible for the reinforcement effects of cocaine) has been under scrutiny due to studies observing that DAT-knockout animals still showed a place preference for cocaine in the conditioned place reference (CPP) test, which is widely used to assess addictive-like behaviour in animals through means of classical conditioning (Bardo and Bevins) (discussed under section 2.6.1) (Hall 2004). This implies that monoamines other than DA play a role in mediating addictive disorders.

2.3.2 Serotonin (5-HT)

There is growing evidence that 5-HT (5-hydroxytryptamine) and dysregulation in the 5-HTergic system plays a role in addiction disorders, especially in the comorbidity of other disorders such as depression during long-term drug abuse (Kirby *et al.*, 2011). Studies show that known drugs of abuse interfere with 5-HT reuptake and release and can result in increased extracellular transmitter concentrations available for binding and activation of 5-HT receptors (Müller *et al.*, 2007).

The 5-HT system is modulated by all major classes of drugs of abuse and contributes to the development of addictive behaviours (Kirby *et al.*, 2011). One of the most popular groups of

drugs of abuse are the psychostimulants, which are known to induce euphoria and arousal in humans by increasing DA and 5-HT (Müller *et al.*, 2007). Animals acutely exposed to cocaine (a widely abused drug), MA, 3,4-methylenedioxymethamphetamine (MDMA), morphine and alcohol have presented with increased 5-HT levels in the frontal cortex, striatum and the hippocampus (Andrews & Lucki, 2001). However, chronic exposure (to the above-mentioned substances) results in variable effects on 5-HT levels; after chronic exposure, amphetamine showed no effect on 5-HT levels in the hippocampus (Barr & Forster, 2011; Barr *et al.*, 2013), morphine decreased 5-HT levels in the frontal cortex and the hippocampus (Mangiavacchi *et al.*, 2001; Goeldner *et al.*, 2011) whilst alcohol decreased the levels in the frontal cortex, striatum and the hippocampus (Burnett *et al.*, 2012). The effects after chronic administration does not seem to follow a specific pattern although the 5-HT transporter (SERT) tends to be up-regulated after chronic exposure to drugs of abuse, so that the higher densities of these transporters increase the reuptake of 5-HT after chronic exposure (Müller *et al.*, 2002). In this instance, place preference was completely eliminated when both the DAT and SERT were knocked out (Hall *et al.*, 2004) and as a result many studies have shifted to the role of 5-HT in drug addiction. Indeed, two 5-HT receptors have been studied intensively for their role in addiction over the past few years, namely 5-HT_{1A} (Müller *et al.*, 2007) and 5-HT_{2A} (Das *et al.*, 2016). These receptors will therefore be discussed specifically below.

5-HT_{1A} receptor

The 5-HT_{1A} receptor shows an 89% similarity in humans and rats, with very high densities of these receptors found in various brain regions (eg. median raphe nucleus, limbic areas, hippocampus, amygdala and limbic areas) (Müller *et al.*, 2007). The 5-HT_{1A} receptor is a subtype of the 5-HT₁ receptor group and is divided into presynaptic auto-receptors and postsynaptic hetero-receptors (Riad *et al.*, 2000; Müller *et al.*, 2007). The activation of both the pre- and post-synaptic receptors follows after 5-HT release from 5-HT neurons (Adell *et al.*, 2002). After the activation of the 5-HT_{1A} auto-receptors, 5-HT cell firing, synthesis as well as the release of 5-HT into the cytosol is diminished (Ago *et al.*, 2003) (See Figure 2-4). Postsynaptic 5-HT_{1A} receptors, located in the limbic system in the brain are of great importance in understanding the effects of known drugs of abuse such as the psychostimulants. 5-HT exerts effects on the DA nuclei of the VTA where it controls cell firing; after the binding of 5-HT (or a 5-HT receptor agonist) to this receptor, DA neurons are depolarized and GABAergic neurons are hyperpolarized leading to increased firing of the DA neurons and increased DA release into the NAcc (see Figure 2-4) (Adell *et al.*, 2002; Ago *et al.*, 2003). Furthermore, activation of the postsynaptic 5-HT_{1A} receptor also has a profound effect on NA release and activity; NA release is increased in the hippocampus after stimulation of these receptors (Riad *et al.*, 2001; Müller *et al.*, 2007).

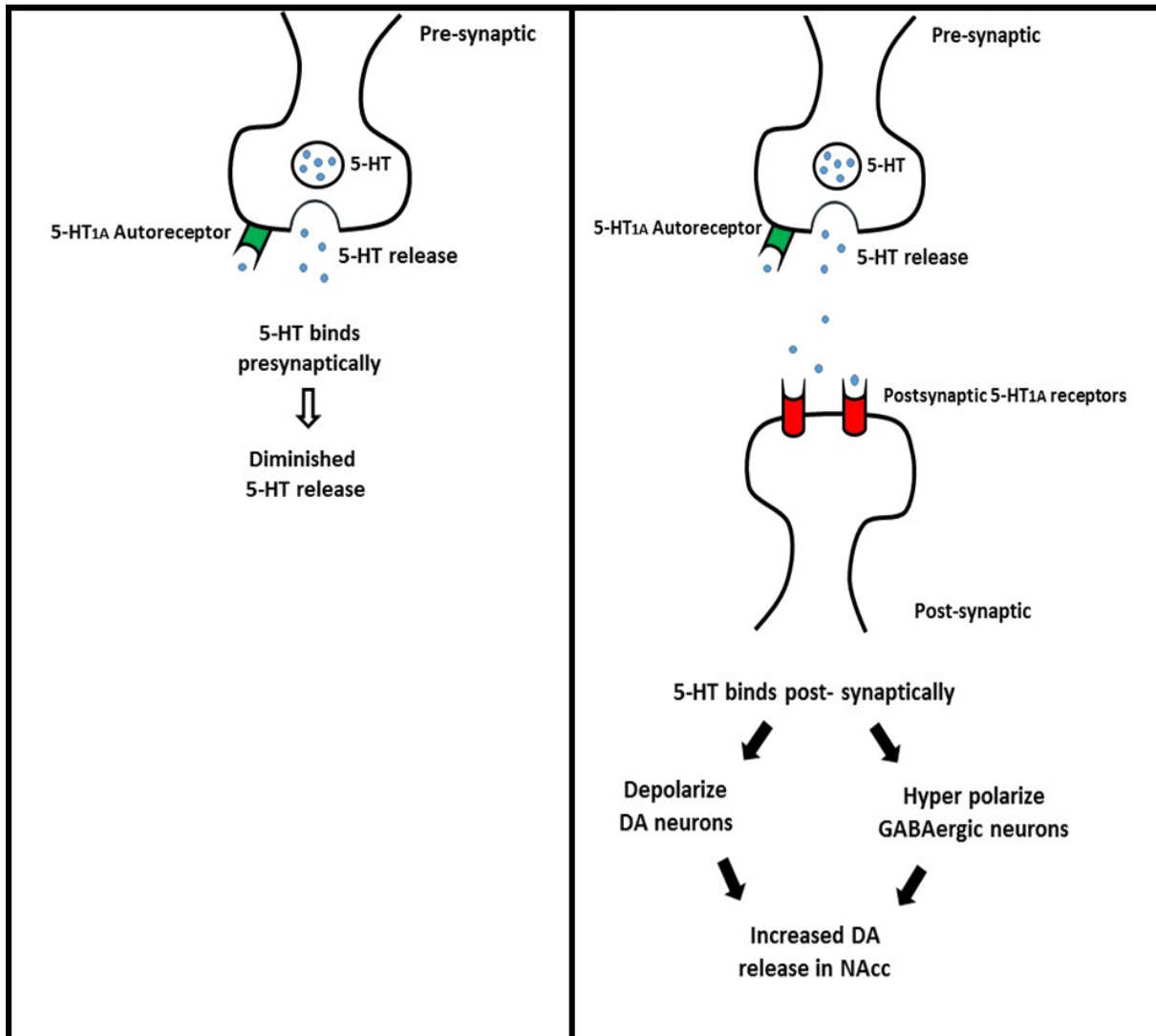


Figure 2-4: Binding of 5-HT on 5-HT_{1A} pre- and post-synaptic receptors. (A) Binding of 5-HT to the pre-synaptic 5-HT_{1A} receptor, diminishing further 5-HT release. (B) Binding of 5-HT to the post-synaptic 5-HT_{1A} receptor, ultimately leading to an increase in DA release in the NAcc (see text for detail) (Adell *et al.*, 2002).

5-HT_{2A} Receptor

The hallucinogens represent a large group of drugs of abuse (of which LSD is the most popular) that alter the state of mind without inducing impairment in memory or causing addiction (Kometer & Vollenweider, 2016). In general, drugs classified as hallucinogens are 5-HT_{2A} receptor agonists (Hollister, 1968; Glennon *et al.*, 1983; Ichikawa *et al.*, 2001), with activation of the 5-HT_{2A} receptor directly responsible for their hallucinogenic effects in humans (Fantegrossi *et al.*, 2008) and surrogate behaviours thereof in rodents (e.g. head twitching / 'wet-dog-shake') (Halberstadt & Geyer, 2013). Moreover, the 5-HT_{2A} receptor is linked to

depression and schizophrenia, where higher densities of these receptors have been found post-mortem in patients that were depressed or committed suicide (Mann *et al.*, 1986), while lower densities of 5-HT_{2A} were observed in schizophrenia patients (Mita *et al.*, 1986). Furthermore, the 5-HT_{2A} receptor is expressed in the same brain regions as the mesocorticolimbic DAergic system, such as the VTA, NAcc and frontal cortex (Bubar & Cunningham, 2007). Here DA neurons are innervated by 5-HT which exert excitatory control over DA neurons (Bortolozzi *et al.*, 2005). Also, there is an interaction between 5-HT_{1A} and 5-HT_{2A} receptors which results in the modulation of DA release in the frontal cortex (Ichikawa *et al.*, 2001; Murat *et al.*, 2017). The activation of 5-HT_{1A} receptors increase the release of DA (through depolarisation as discussed earlier), which is potentiated by 5-HT_{2A} receptor blockade leading to increase DA release (Ichikawa *et al.*, 2001).

Because of the high affinity of 5-HT for its pre-and postsynaptic receptor, the involvement of 5-HT in the behaviour, emotion and memory processes underlying drug addiction, as well as the comorbidity of depression and anxiety in drug addiction, are to be expected (Müller *et al.*, 2007; Kirby *et al.*, 2011; Halberstadt & Geyer, 2013).

2.3.3 Noradrenaline (NA)

NA is one of the most abundant neurotransmitters in the brain and plays a significant role in learning and memory (Gibbs & Summers, 2002). The two main NA projections in the brain innervate the hippocampus, cerebellum and the forebrain through projections originating from the dorsal locus coeruleus (LC), as well as the hypothalamus, midbrain and the amygdala via the ventral LC (reviewed Weinshenker and Schroeder (2007)). The innervation to many of these regions of the brain underlies the ability of NA to control many of the aspects associated with drug addiction, including reward and relapse (Gibbs & Summers, 2002).

The fact that many drugs of abuse such as the amphetamines increase levels of NA by either blocking its reuptake or increasing its release has promulgated the idea that NA is a major contributor in the biology of addiction and drug abuse (Wise, 1978; Wise, 2004a). However, some studies do not provide support to this theory, since the drugs that have an effect on NA (such as the amphetamines) also have a profound effect on DA; indeed, selectively blocking the DA receptors in rodents significantly diminished self-stimulation (Wise, 1978; Weinshenker & Schroeder, 2007). Furthermore, although locomotor activity is not a test to measure addictive-like behaviour per se, most preclinical studies describe drugs of abuse (including alcohol, cocaine and the opiates) as inducing hyperlocomotion (reviewed in Weinshenker and Schroeder (2007)), which is mediated by the DAergic and NAergic systems (Robinson & Berridge, 2000). Drug-induced hyperlocomotion has been found pre-clinically, to be attenuated by the administration of an α_{1a} receptor blocker (Mohammed *et al.*, 1986;

Weinshenker *et al.*, 2002), while α_{2a} pre-synaptic receptor blockers elevate NA levels and augment stimulant-induced hyper-locomotion (Villégier *et al.*, 2003).

2.3.4 GABA and glutamate

In the brain, GABA and glutamate functions as the main inhibitory and excitatory neurotransmitters respectively (see Figure 2-2) (Petroff, 2002). In a preclinical study by Schilström *et al.* (1997), animals received a glutamate receptor antagonist which mostly blocked the stimulatory effects on DA neurons (ultimately leading to a decrease in DA release) in the VTA, indicating that the glutamatergic input from the frontal cortex plays a significant role in the modulation of DA release in the VTA. This is in line with other studies, indicating that DA activity in the VTA is regulated by the activation of NMDA and non-NMDA glutamate receptors situated on DA neurons in the VTA (Kalivas *et al.*, 1989; Kalivas & McFarland, 2003). After the activation of the frontal cortex, glutamatergic afferents projecting from there to the VTA induces the firing of the DA neurons and thereby increases the concentration of DA in the NAcc (Murase *et al.*, 1993; French, 2016). A vast amount of studies suggest that drug seeking behaviour is abolished when glutamate receptors are blocked (Kenny & Markou, 2004; McFarland *et al.*, 2004), while conversely, an increase in frontal cortical glutamate release is associated with augmented drug-seeking behaviour (McFarland *et al.*, 2004). These findings have prompted the search for glutamatergic drugs in the treatment of drug addiction (Bisaga & Popik, 2000); for instance, NMDA receptor antagonists such as memantine, dextromethorphan, amantadine, ketamine and phencyclidine are hypothesized to mediate and alter the adaptive processes implicated in the development and maintenance of drug addiction (Bisaga & Popik, 2000). This novel approach may diminish withdrawal effects, and normalize the changes induced by addiction and its ensuing neurochemical changes. Pre-clinical studies have also indicated that NMDA receptor antagonists successfully attenuate the self-administration of cocaine (Ranaldi *et al.*, 1997; Panagis *et al.*, 2000) and conditioned learning induced by morphine, cocaine and methamphetamine (Schulteis *et al.*, 1997).

2.3.5 Endocannabinoids

As discussed earlier, most drugs of abuse have a common mechanism through which they exert their neurobiological effects, specifically via actions on the mesocorticolimbic DAergic, NAergic and 5-HTergic pathways (Goldstein & Volkow, 2002; Goodman, 2008). However, the endocannabinoid (EC) system is also involved in modulating monoaminergic transmission (Melis *et al.*, 2004).

In this regard, two receptors have been identified, namely the cannabinoid-1 (CB₁) and CB₂ receptors which acts as the binding site for the ECs, anandamide (Devane *et al.*, 1992; Piomelli, 2003) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995; Piomelli, 2003).

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The ECs act as messengers in the CNS where they play a role in modulating synaptic transmission. They are especially responsible for the rewarding and addictive-like effects of many drugs of abuse such as marijuana and other cannabinoids (Lichtman & Martin, 2005) and participate in the addictive properties of these drugs (Melis *et al.*, 2004).

ECs are known to be retrograde messengers, which implies that instead of traveling from pre- to post synaptic terminals, these neurotransmitters are released post-synaptically from terminals (such as DA and glutamate) when such neurons are firing in high frequency bursts (Melis *et al.*, 2004). In the case of a DA neuron, high frequency bursts lead an increase in Ca^{2+} release and activation of EC synthesizing enzymes (viz. diacylglycerol lipase (DGL)) to produce 2-AG which in turn binds to pre-synaptic CB_1 receptors (Melis *et al.*, 2004; Jung *et al.*, 2005; Wang & Lupica, 2014). This ultimately leads to the modulation of excitatory (glutamatergic) and inhibitory (GABAergic) neuro-transmission. These effects are evident especially in the VTA and NAcc where CB_1 receptors are most abundant (Katona *et al.*, 2001). Thus, activation of CB_1 receptors on the terminals of the GABAergic neurons in the VTA indirectly inhibit the inhibiting effects of GABA on DAergic neurons, thereby increasing the firing rates of the DA neurons in this region of the brain (Katona *et al.*, 2001; Melis *et al.*, 2004) (see Figure 2-5). The increased activity of DA neurons induces further release of ECs from the DA terminals, thereby inhibiting both GABA and glutamatergic inputs to the DA neurons in the VTA (Melis *et al.*, 2004) (see Figure 2-5). Cannabinoids also increase the concentrations of glutamate in the frontal cortex and decrease GABA transmission in the hippocampus leading to memory and learning deficits (Ferraro *et al.*, 2001).

2.3.5.1 Role of the endocannabinoid system in drug seeking:

As discussed earlier, drugs of abuse (including cocaine, ethanol and nicotine) increase the levels of DA in the brain through increased firing of these neurons (Cheer *et al.*, 2000; Goldstein & Volkow, 2002; Melis & Pistis, 2012). When DA is released in a phasic manner, conditioned learning is made possible through associations made with the environment which later leads to drug-seeking (Cheer *et al.*, 2007). EC receptor blockers, such as rimonabant, diminish the firing of DA neurons (through the inhibitory effects of EC on GABA release) (Le Foll & Goldberg, 2004), resulting in a net decrease in DA neuron firing. This indicates a promising avenue for possible intervention with regards to the treatment of addiction and drug seeking behaviours.

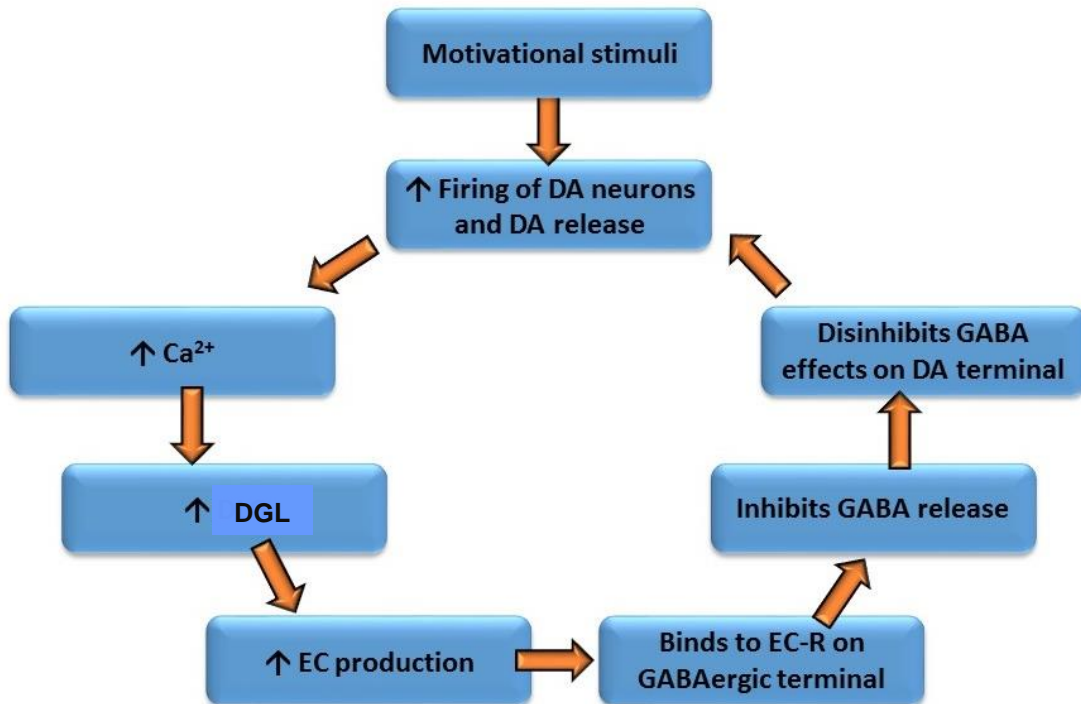


Figure 2-5: Role of the endocannabinoid system in drug seeking When motivational stimuli are presented (like drug-related cues) the firing of DA neurons are increased and through endocannabinoid signalling, disinhibits the effects of GABA on the DA leading to higher levels of DA. (Adapted from: (Oleson & Cheer, 2012)) (See section 2.2.5 for details). Abbreviations: DA, dopamine; Ca²⁺, calcium; DGL, diacylglycerol lipase; EC, endocannabinoid; EC-R, endocannabinoid receptor; GABA, gamma-aminobutyric acid.

2.4 Oxidative stress

As discussed earlier, most drugs of abuse (such as MA and cocaine) induce their effects by exerting an effect on DA transmission in the mesocorticolimbic regions of the brain (Goldstein & Volkow, 2002) (as discussed above). Since addiction is seen as a brain disease, neurotoxicity may be the reason for some of the effects of these drugs (Leshner, 1997). Neurotoxicity is mainly observed in brain areas where DA nerve terminals are abundant, as is known to occur after the use of drugs of abuse such as MA (reviewed in Cunha-Oliveira *et al.* (2008)). Further *in vitro* and *in vivo* studies also indicate the neurotoxic effects of DA by inducing oxidative stress, especially in DA rich brain areas (Coyle & Puttfarcken, 1993; Andersen, 2004). DA is metabolized by monoamine oxidase to produce 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide (H₂O₂), while it also undergoes auto-oxidation to form superoxide anion (O₂⁻) and other quinone derivatives (see Figure 2-6) (Cunha-Oliveira *et al.*, 2008). If the antioxidant systems of the body are unable to control the

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levels of reactive oxygen species (ROS) it may lead to cell death in these regions as well as the neighbouring cells (Cunha-Oliveira *et al.*, 2008) (see Figure 2-6). This elevation of oxidative stress may ultimately be the cause of neurotoxicity associated with drugs of abuse (Cunha-Oliveira *et al.*, 2008). Studies have not only observed that DA plays a role in the production of ROS, but of the role of glutamate in the production thereof (Atlante *et al.*, 2001; Parfenova *et al.*, 2006). An increase in glutamate levels are directly associated with an increase in ROS, lipid peroxidation and subsequent apoptosis (Parfenova *et al.*, 2006; Sadeghnia *et al.*, 2017).

Studies show that oxidative stress and inflammation is becoming increasingly relevant in the abuse of known drugs of abuse such as cocaine (Kovacic, 2005). Previous clinical and pre-clinical studies have also observed a significant increase in CNS ROS as well as lipid peroxidation after MA exposure (Cunha-Oliveira *et al.*, 2008), while rat studies have shown that MA decreases glutathione (GSH) levels, the antioxidant responsible for the inactivation of H₂O₂ (Korciem *et al.*, 2013). Clinical studies by Fitzmaurice *et al.* (2006) also concluded that oxidative damage is present in the brain tissue of MA abusers. Furthermore, preclinical studies observed that rats exposed to cocaine had a significant increase in H₂O₂, lipid peroxidation, SOD and lower catalase activity in the frontal cortex (Dietrich *et al.*, 2005; Bashkatova *et al.*, 2006) as well as increased lipid peroxidation in the hippocampus and NAcc, due to impairment of endogenous anti-oxidant systems (Macêdo *et al.*, 2005).

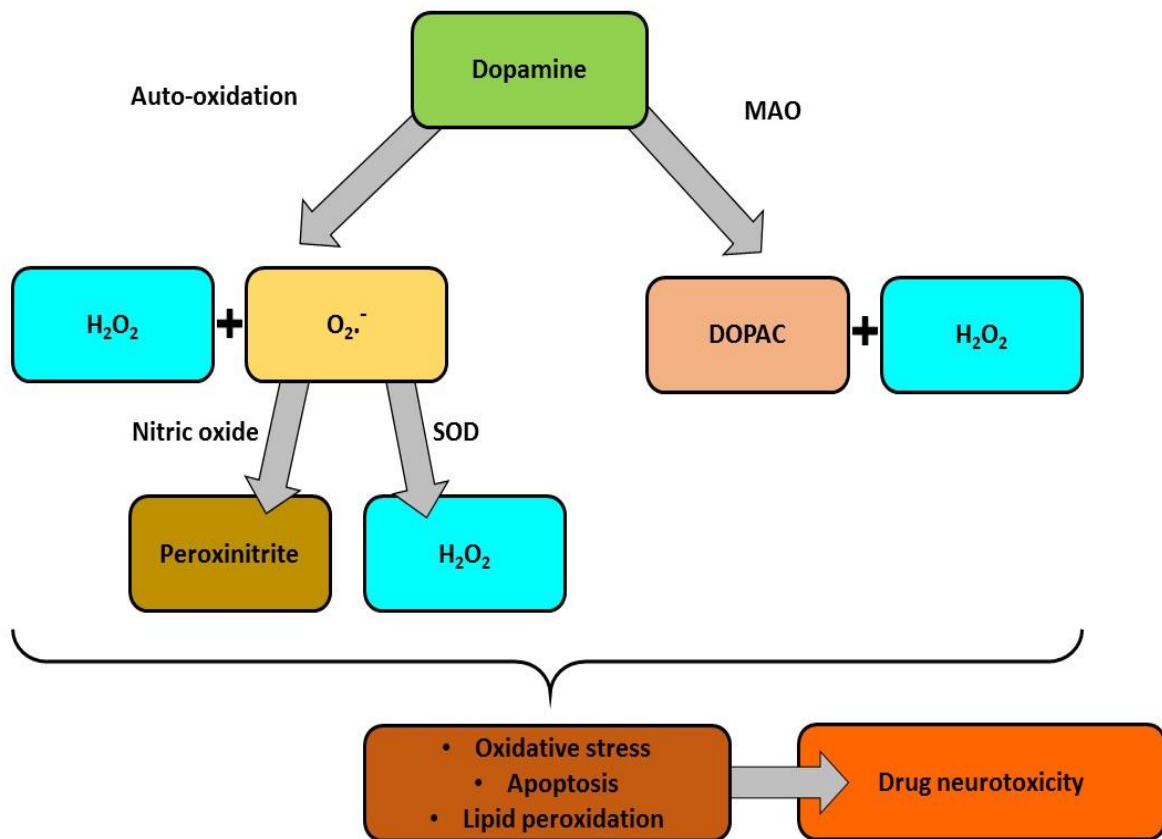


Figure 2-6. Metabolic pathways of DA leading to oxidative stress. Metabolism of dopamine (DA) by monoamine oxidase (MOA) and auto-oxidation leads to an increase in hydrogen peroxide H_2O_2 and superoxide anion O_2^- . O_2^- is detoxified by superoxide dismutase (SOD) to H_2O_2 . O_2^- reacts with nitric oxide and delivers a toxic compound, peroxynitrite. If the antioxidant system is unable to decrease the increased levels of ROS in the brain, lipid peroxidation will occur leading to cell death and damage in these regions, linked to the neurotoxicity of drugs of abuse.

2.5 A brief discussion on known drugs of abuse

Only the drugs of abuse relevant to this study will be discussed below, viz. marijuana, the most common drug of abuse worldwide and one of the main constituents of nyaope (Oleson & Cheer, 2012; Rough *et al.*, 2014), MA, the positive control in this study, and LSD, the main psychoactive drug that has shown a common mechanism of action with efavirenz and the latter's penchant to induce neuropsychiatric effects (Gatch *et al.*, 2013).

2.5.1 Marijuana

Following administration of marijuana (also known as “dagga” in South Africa), DA release in the NAcc is increased due to an increased firing rate of DAergic neurons in the VTA (Pierce & Kumaresan, 2006). Marijuana is the common name for *Cannabis sativa*, of which Δ^9 -tetrahydrocannabinol (Δ^9 -THC) is the psychoactive agent (Pistis *et al.*, 2002). The binding of Δ^9 -THC to CB₁ receptors on inhibitory GABAergic terminals in the VTA disinhibits DAergic neuronal activity resulting in increased DA release in the NAcc (Cheer *et al.*, 2004; Pierce & Kumaresan, 2006) (see Figure 2-5). The psychoactive profile of marijuana includes euphoria and enhancement of visual and auditory perception referred to as the “high”, while impairment of memory, dysphoria and anxiety induces the so-called “stoned” profile (Pistis *et al.*, 2002). Moreover, high doses of marijuana are reported to cause sedation (Pistis *et al.*, 2002). As mentioned earlier, anandamide is an EC neurotransmitter that exhibits partial agonist activity on the CB₁ receptors in the central and peripheral nervous system (Leishman *et al.*, 2016). Rodent studies have found anandamide to enhance the pleasurable responses to a rewarding sucrose taste, and to enhance food intake (Pacher *et al.*, 2006). Another study by Diana *et al.* (1998) also observed dense CB₁ receptor expression in the frontal cortex of healthy animals where cannabinoids such as Δ^9 -THC act to increase DAergic signalling through the above-mentioned process.

2.5.2 Methamphetamine (MA)

Methamphetamine (methamphetamine/metamfetamine/N-methyl-1-phenylpropan-2-amine) addiction is a serious problem worldwide, affecting certain age and social groups more than others (Cruickshank & Dyer, 2009). This drug is available in many forms including pure crystalline forms, tablets or hydrochloric salts and is taken through sniffing, inhalation, oral or intra venous administration (Barr *et al.*, 2006). MA is the second most popular drug of abuse in the world and is known to cause many adverse effects in the user (Cruickshank & Dyer, 2009).

By modulating the DAT, the NA-transporter (NAT) and the SERT as well as the vesicular monoamine transporter-2 (VMAT-2), MA acts as an indirect agonist at DA, NA and 5-HT receptors (Cruickshank & Dyer, 2009), having structural similarities to these monoamines. After MA administration, monoamines are redistributed and released into the cytosol, this being achieved by reversing the effects of the above transporters through the disruption of the pH gradient that would normally lead to accumulation of monoamines in the vesicles (Cruickshank & Dyer, 2009), leading to the release of stored monoamines in the cytosol and a resultant stimulation of post synaptic receptors (Cruickshank & Dyer, 2009). Another mechanism through which MA exerts its effects is by diminishing monoamine metabolism

through the inhibition of monoamine oxidase (MAO), resulting in increased synaptic monoamine concentrations (Sulzer *et al.*, 2005).

The clinical effects of MA include: arousal (racing mind and reduced fatigue) (Perez-Reyes *et al.*, 1991) facilitated by the stimulation of adrenoceptors in the forebrain (Berridge, 2006); euphoria (feeling high and intoxicated) (Mendelson *et al.*, 2006) mainly facilitated by the DA release in the NAcc (Völlm *et al.*, 2004); relaxation (feeling confident with decreased tension) (Mendelson *et al.*, 2006) through stimulation of 5-HT₁ receptors, accounting for the anxiolytic effects (Filip *et al.*, 2005); anxiety (dysphoria and nervousness) possibly due to increased adrenoceptor stimulation; as well as talkativeness, paranoia, visual and auditory hallucinations (Völlm *et al.*, 2004; Mendelson *et al.*, 2006), likely due to its stimulatory effects on D2 receptors. Physiological effects include: an increase in heartrate due to increased stimulation of the β -receptors in heart tissue; increased blood pressure via α 1-receptor activation; along with increased respiration, elevated body temperature, pupil dilation, reduced appetite, improved attention span and an increase in coordination (Fotiou *et al.*, 2000; Brooks, 2003; Halford *et al.*, 2004; Mills *et al.*, 2004; Hart *et al.*, 2008). Prolonged MA exposure has been observed to decrease DA levels in the striatum of humans (Wilson *et al.*, 1996).

2.5.3 Lysergic acid diethylamide (LSD)

LSD was synthesized in 1938 and is classified as one of the most potent hallucinogens (Kometer & Vollenweider, 2016), originally indicated for use in psychotherapeutic treatment in 1960's. However, shortly thereafter its recreational use for spiritual and psychedelic purposes followed (Passie *et al.*, 2008). After acute exposure to very low dosages of LSD, psychotic behaviour precipitates which includes an array of mind altering effects including a slowing of time, "seeing" sound, recalling vivid memories, visual deceptions and a feeling of meaningfulness and deep insight in the user (Passie *et al.*, 2008). LSD mediates these effects without increasing 5-HT, but rather by binding primarily to 5-HT_{2A} receptors (see section 2.2.2), with a higher affinity than 5-HT, on glutamate neurons in the thalamus (Hollister, 1968; Glennon *et al.*, 1983; Kometer & Vollenweider, 2016). The resulting change in serotonergic transmission leads to an increase in glutamate release, ultimately inducing hyper-glutamatergic transmission in the frontal cortex and subsequent down-stream effects on DA release in the striatum (see Figure 2-7). As noted earlier binding of glutamate on the NMDA receptors in the frontal cortex leads to an increased firing of DA neurons in the VTA thereby increasing DA release in the NAcc. The increased glutamate release in these regions may also contribute to cell death as the result of increased ROS and lipid peroxidation (Sadeghnia *et al.*, 2017) Although LSD is illegal, it is not classified as an addictive drug since the use of the drug does not lead to compulsive use (Hyman & Malenka, 2001).

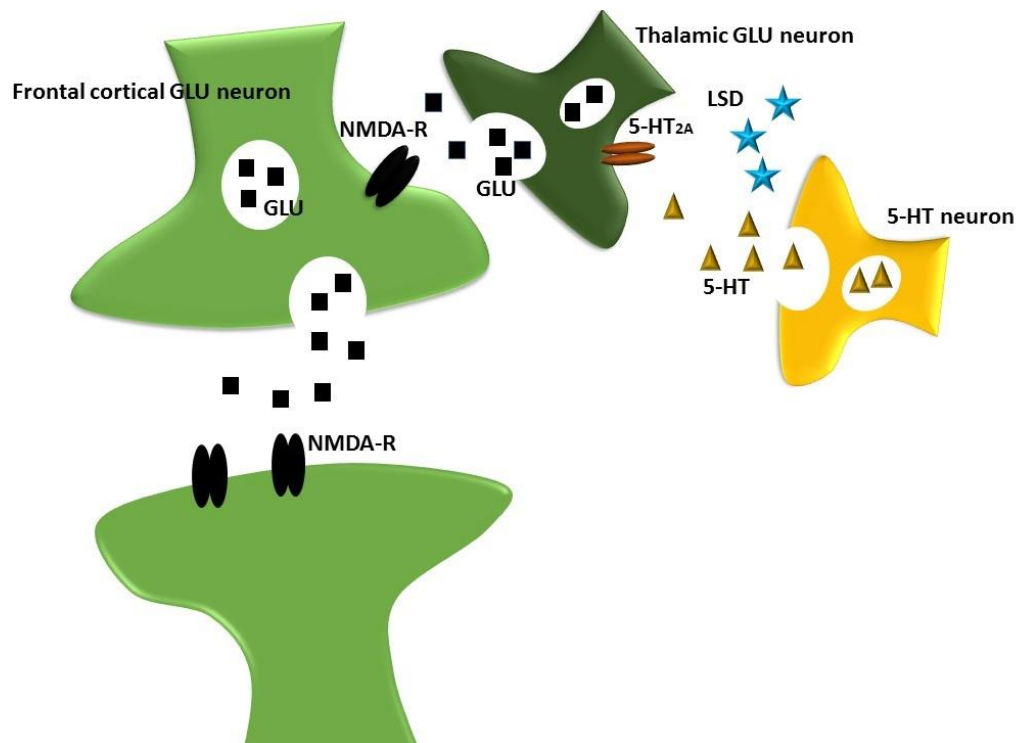


Figure 2-7: LSD mediated alteration in neurotransmission (adapted from (Ham *et al.*, 2017)) *5-HT*: serotonin, *LSD*: lysergic acid diethylamide, *GLU*: glutamate, *NMDA-R*: N-methyl-d-aspartate receptor. LSD mediates these effects by 5-HT_{2A} receptor binding ultimately leading to increased DA release. (See text for details)

2.6 Preclinical research in addiction

“The public questions why animals are made to ‘suffer’ for a problem that people inflict on themselves. Animal rights extremists may exploit this sentiment as they attempt to generate opposition to this area of research. However, addictive diseases and their comorbid clinical conditions (such as HIV, hepatitis C, cirrhosis) are biomedical diseases with massive personal and societal costs, and brain structures and responses often are chronically affected in long-term addicts.”

- Lynch *et al.* (2010)

Thus, to better understand the mechanism underlying the abuse of drugs on a neurochemical and behavioural level, animal models have proven to be valuable in acquiring new knowledge on the pathophysiology of substance abuse disorders (Lynch *et al.*, 2010). Although animal models are insufficient to provide exact mechanism involved in the abuse of drugs in humans, it may improve our understanding of underlying mechanism through which drugs elicit their

effects. Reproduction of addiction in a laboratory setting is impossible since drug abuse is a phenomenon only seen in humans. However it is possible to model some behaviour in experimental animals that can be translated to drug abuse in humans (Kalivas & McFarland, 2003; Planeta, 2013). Many behavioural models such as the CPP test (Bardo & Bevins, 2000), SPT (Tönissaar *et al.*, 2006), self-stimulation (Gatch *et al.*, 2013) and locomotor activity in the OFT (Prut & Belzung, 2003) are widely used. Animal studies on drugs of abuse are of great importance because, in contrast to clinical studies, variables in the test population can easily be controlled for (Lynch *et al.*, 2010). Thus, these studies focus on the primary effects and the ability of certain drugs to directly alter the behaviour of the subjects and to compare these changes to the definition of addiction on a behavioural level (Lynch *et al.*, 2010). The effects of these drugs involve processes that are common in all mammalian species (Bozarth, 1990). Although clinical models provide exceptional data for evaluation and assessment of drugs and effects thereof, some drugs such as LSD and MA are prohibited in testing in humans due to various ethical concerns (Festing & Wilkinson, 2007). Furthermore the effects of these drugs on the neurochemistry of the abuser are difficult to assess, thus making pre-clinical trials a necessity (Lynch *et al.*, 2010).

The above-mentioned behavioural tests performed in preclinical studies evaluating drugs of abuse and relevant to this study will now be discussed in more detail.

2.6.1 Conditioned place preference test (CPP)

Among all the preclinical tests to evaluate the rewarding properties of a drug in animals, the CPP test is one of the most popular and widely used (Bardo & Bevins, 2000). In this paradigm, making use of classical Pavlovian conditioning, the motivational or aversive properties of a drug serves as an unconditioned stimulus that is repeatedly paired with a neutral environmental stimulus which eventually leads to the previously neutral environmental stimulus being associated with the drug and thus acts as a conditioned stimulus (Bardo & Bevins, 2000; Tzschentke, 2007).

Spragg (1940) did a study that is seen as one of the earliest forms of CPP. In that study chimpanzees were given doses of morphine and after becoming addicted to the drug the animals were trained to choose between a box with a syringe filled with the daily morphine dose and another box with a food reward. The animals chose the syringe box on days they were deprived of morphine and the food reward on days that they have been pre-treated with morphine. Although many variations in design and methodology occur in different laboratories, the basic principles remain the same: the stimulus of choice (e.g. the potentially rewarding or aversive drug) is administered and paired with a distinctive compartment, visually and

texturally different from a vehicle paired compartment. The compartment contexts vary in flooring, size or shape, wall colour or pattern and olfactory cues (Bardo & Bevins, 2000).

2.6.1.1 CPP Methodological considerations:

The most common variation of the paradigm is the three compartment apparatus which has two outer chambers (visually and texturally different) connected by a smaller centre compartment with no special characteristics (see Figure 2-8), or a two compartment model with no centre compartment (Prus *et al.*, 2009). The important difference is that with a two compartment apparatus, the animals have a forced choice between either of the two compartments. The concern here is the bias of the animal toward the chamber that it was placed in at the beginning of the session (Prus *et al.*, 2009), whereas the three compartment apparatus allows the animal access to a central compartment between the chambers used in the experiment, thus avoiding such bias.

Another consideration in the paradigm is the use of a biased or unbiased design. When subjects are tested before conditioning, some animals will prefer compartment A and others will prefer compartment B. The biased research design takes this into account, so that during the habituation period (discussed further on) the preference of each animal is assessed and the least preferred compartment is assigned the drug-paired compartment. In an unbiased design the compartments are assigned by the researcher prior to conditioning (Bardo & Bevins, 2000; Prus *et al.*, 2009). A study by Calcagnetti and Schechter (1994) using the biased design showed that animals who received nicotine and subjected to their least preferred compartment showed an increase in time spent in that compartment on days of testing whereas animals who were subjected to their most preferred compartment showed neither a place preference or aversion after conditioning with nicotine. For this reason, many studies have made use of the biased study design (Cunningham *et al.*, 2003; Biala & Budzynska, 2008; Baracz *et al.*, 2012).

The CPP paradigm consists of 3 phases:

a) Habituation:

During the habituation period animals are allowed to roam freely between all three compartments of the apparatus, this is to allow the animal to habituate to the environment and to rule out novelty as a variable (Cunningham *et al.*, 2003; Prus *et al.*, 2009). During this period, the total amount of time spent in each compartment is quantified and the compartment in which the least amount of time was spent is assigned as the drug-paired compartment.

b) Conditioning

During the conditioning period, which last anything from 6-12 days (Cunningham *et al.*, 2003; Prus *et al.*, 2009; Gatch *et al.*, 2013), animals are injected with the possible rewarding or aversive drug and subjected to the drug-paired compartment for a period of 5-60 min, depending on the drug, for three to four sessions. The number of sessions depends on the sensory differences in the compartments as well as the potency of the drug's rewarding properties (Cunningham *et al.*, 2003; Prus *et al.*, 2009). On alternating days, animals receive only vehicle and subjected to the vehicle-paired compartment for 5-60 min for three to four sessions. The amount of conditioning sessions and conditioning times are decided upon at the discretion of the researcher.

c) Post conditioned testing

Following conditioning, the rewarding or aversive effects of a drug can be assessed by allowing the animal free access to all the compartments where increased time spent in the drug-paired compartment serves as a CPP and evidence of a rewarding unconditioned stimulus (drug) (Bardo & Bevins, 2000; Prus *et al.*, 2009).

2.6.1.2 Other considerations in the CPP test

Given the motor retarding action of some drugs, some protocols for tests such as the forced swim test require a pre-test to assess non-specific effects on locomotor activity prior to testing their behaviour in the swim test (Slattery & Cryan, 2012). In this instance, locomotor activity may influence the results acquired in the CPP test since mobility of the animal is crucial in identifying a place preference. Thus, drugs that decrease the locomotion of the animal may ultimately lead to immobility that can lead to a false positive or negative response in the CPP test.

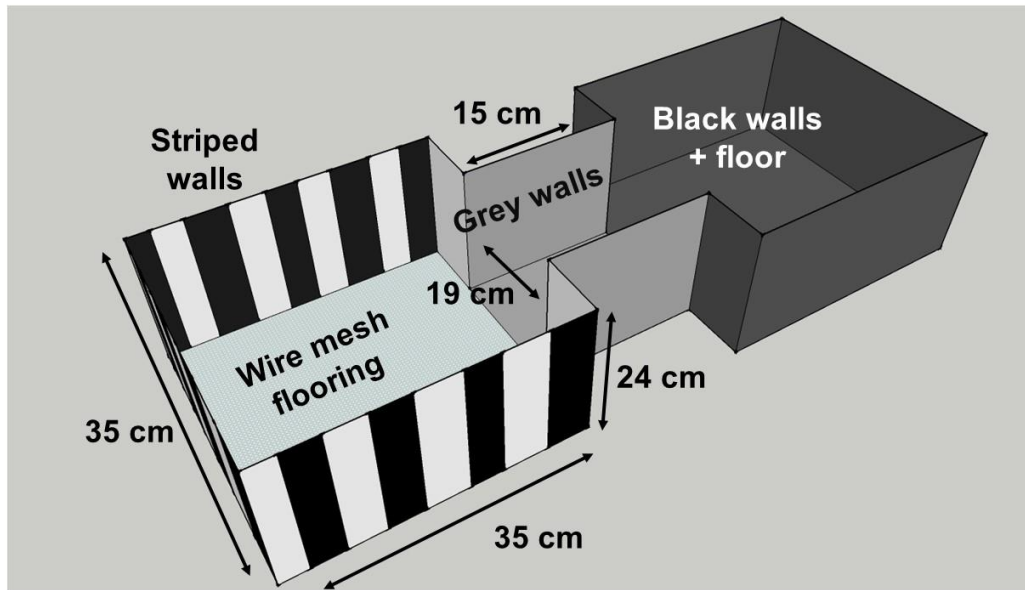


Figure 2-8: Illustration of the three compartment CPP apparatus. (See text for details)

2.6.1.3 Known drugs of abuse and the CPP

Many drugs of abuse have been used in CPP. However, for the purpose of this study, the drugs and the mechanisms through which they produce a CPP will be limited to psychostimulants (such as MA), hallucinogens (such as LSD), cannabinoids and efavirenz. The relevance of these drugs being that marijuana is the most common drug of abuse worldwide and one of the main constituents of nyaope, MA is used as a positive control in this study and LSD is the main psychoactive drug with which the mechanism of efavirenz's induced neuropsychiatric effects has been compared (Gatch *et al.*, 2013). Since efavirenz is being studied for addictive or rewarding properties, a discussion of previous articles on this subject is also included.

Cannabinoids

As mentioned previously, Δ^9 -THC is one of the bioactive cannabinoids found in marijuana, and increases DA levels in the NAcc due to the effects it exerts on CB1 receptors, leading to the disinhibition of DA neurons (Pistis *et al.*, 2002; Pierce & Kumaresan, 2006) (discussed earlier in section 2.3.5). A preclinical study done by Braida *et al.* (2004) assessed the rewarding effects of Δ^9 -THC in the CPP at doses of 0.015, 0.075, 0.15, 0.37, 0.75, 0.75, 1.00, 3.00, and 6.00 mg/kg in male Wistar rats, and observed that doses between 0.075 and 0.75 mg/kg produced a significant increase in the time spent in the drug-paired compartment compared to vehicle treatment. However, at higher doses the animals spent less time in the drug-paired compartment which indicates a conditioned place aversion at higher doses. Similarly, another study (Cheer *et al.*, 2000) also observed place aversion in the CPP test after administration of 1.5 mg/kg Δ^9 -THC to rats.

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Psychostimulants

Psychostimulants known to induce a place preference in the CPP include MA (at 1mg/kg) (Zakharova *et al.*, 2009) and cocaine (15 mg/kg i.p) (Masukawa *et al.*, 1993; Prast *et al.*, 2014). These drugs produce a significant CPP through their effects on the limbic system, of which DA is a primary neurotransmitter (Kalivas & McFarland, 2003). As previously stated, cocaine is a potent inhibitor of the reuptake of DA, 5-HT and NA, so that through increasing DA levels, cocaine is able to induce a CPP (Nazarian *et al.*, 2004; Prus *et al.*, 2009). A study done by Brabant *et al.* (2005) tested the influence of dose and the number of times the drug is paired with the drug-paired compartment and found that two and four conditioning sessions showed similar results in producing a CPP. The effects of MA observed in the CPP paradigm is also due to increasing DA concentrations, as discussed earlier (Prus *et al.*, 2009; Karila *et al.*, 2010).

Hallucinogens

As discussed earlier under section 2.4.3, the well-known drug of abuse, LSD, mediates its effects by primarily interacting with glutamate transmission in the frontal cortex through binding to 5-HT_{2A} receptors (Komater & Vollenweider, 2016). This drug does not show any reinforcing effects in monkeys, and in rodents only very low dosages of LSD have been shown to increase the time spent in the drug-paired compartment after conditioning (Meltzer *et al.*, 1977; Gatch *et al.*, 2013). Another study found that sex plays a crucial role in the ability of LSD to mediate its rewarding effects, observing that only male rats indicated a place preference after conditioning with 0,2 mg/kg i.p (Meehan & Schechter, 1998; Goodman, 2008; Goodwin, 2016).

Efavirenz

The effect of efavirenz in the CPP has only been evaluated recently (Gatch *et al.*, 2013), with a total of eight conditioning sessions (four drug pairings). This study made use of an unbiased study design (discussed earlier), 15-minute conditioning sessions and dosages ranging from 5 mg/kg to 10 mg/kg for the first two sessions and then 20 mg/kg for the second two drug conditioning sessions. Neither a preference nor aversion were obtained during the post-test, although the authors concluded that the outcomes of the CPP did not rule out the possibility that efavirenz may have addictive or rewarding effects and that under different testing conditions (CPP methodology and dosing), efavirenz might produce a rewarding and addictive-like behavioural response in the CPP.

2.6.2 Open field test (OFT)

The psychomotor stimulant theory of drug abuse and addiction suggests that positive reinforcing drugs such as alcohol, opiates, and stimulants induce an increase in locomotor activity through a common mechanism of altering DA projections to the forebrain (Müller *et al.*,

2007; Weinshenker & Schroeder, 2007). The OFT is used to assess the locomotor activity of a subject, although locomotor activity per se is not strictly a test to assess drug reward. Nevertheless, most drugs of abuse such as MA increase locomotor activity, presenting with face validity (hyper-locomotion). Consequently, this test is widely used to assess and predict the rewarding properties of a drug, as evinced by a number of investigators (Mohammed *et al.*, 1986; Villégier *et al.*, 2003; Weinshenker & Schroeder, 2007; Vanderschuren & Pierce, 2010; Aarde *et al.*, 2013). As mentioned earlier under section 2.6.1.2, locomotor activity may influence the results acquired in the CPP since mobility of the animal is crucial in identifying a place preference, thus the OFT may be a valuable test to validate findings in the CPP.

2.6.3 Sucrose preference test (SPT)

The SPT is generally used to measure anhedonia in experimental animals and the ability to experience pleasure (Der-Avakian & Markou, 2012). The test is widely used in studies assessing the effects of antidepressants and anti-psychotics, although can also be a valid measure of reward deficits in other psychiatric disorders since the same circuits are involved (Der-Avakian & Markou, 2012). One system in particular which contributes significantly to the experience of pleasure and reward is the EC system (Melis & Pistis, 2012). In the NAcc the activation of these receptors leads to an increased intake of sucrose in animals (Berridge & Robinson, 1998). The D₁ receptor is especially associated with the rewarding nature of food as well as the reinforcement of food palatability on place preference (Der-Avakian & Markou, 2012) When DA receptor blockers are administered to animals the response to reward is decreased (Markou *et al.*, 1998; Haney *et al.*, 2001). The same is observed when drugs of abuse such as the psychostimulants are discontinued; these drugs decrease the amount of DA in the NAcc and lead to various symptoms including anhedonia (Markou *et al.*, 1998; Brady & Sinha, 2007).

Although not strictly a test to measure addictive-like behaviour, this simple test may show whether or not certain drugs of abuse interact with reward pathways in the brain to promote and/or indulgence in pleasurable activities (Der-Avakian & Markou, 2012), in this instance the hedonic effect evoked by a pleasurable taste. This test is easily carried out in the cages of the animals where animals are individually housed (Rygula *et al.*, 2005; Brigman *et al.*, 2010). In the cages animals have food ad libitum with an addition of two identical bottles, one filled with normal tap water and the other with a sucrose solution (see Figure 2-9). Bottles are changed after 12 hours to rule out a possible side-preference when drinking. The total amount of sucrose solution and water consumed are measured and sucrose consumption is expressed as a percentage of total fluid consumption (Rygula *et al.*, 2005).

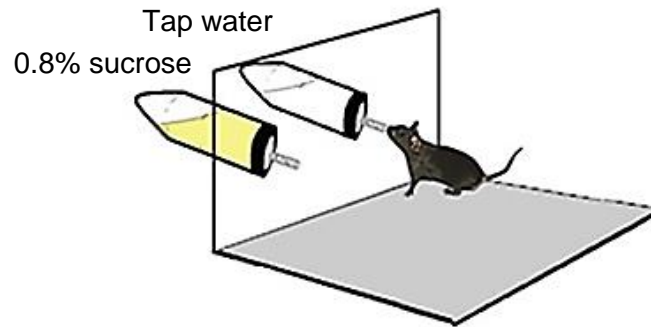


Figure. 2-9: Setup for the sucrose preference test in measuring anhedonia. Animals are subjected to normal tap water and a 0.8% sucrose solution for 24 hours (adapted from: (Brigman *et al.*, 2010).

2.7 Synopsis

Drug abuse is defined as the use of a drug that is not in accordance with the approved manner of use of the specific drug, whereas drug dependence is seen as a complex disease of the brain that results in constant and overwhelming intake of a drug despite harmful consequences (Koob & Le Moal, 1997; Koob, 2000; Goodman, 2008). Many theories on drugs of abuse exist, with the most popular being the incentive sensitization theory, which suggests that stimuli in the environment (unconditioned stimulus) can become a conditioned stimulus after continuously being paired with a drug through means of classical conditioning (Koob, 2006; Robinson & Berridge, 2013). This is important since drug related cues can lead to cravings and a need to administer the specific drug (Robinson & Berridge, 2013).

Drugs that are regularly abused include nicotine, alcohol, marijuana, MA and LSD (Pistis *et al.*, 2002; Passie *et al.*, 2008; Cruickshank & Dyer, 2009). However, recent news reports in South Africa have surfaced describing people crushing and smoking a white powder known to consist of anything from milk powder, heroin, pool cleaner and cocaine (Ngaimisi *et al.*, 2010; Fihlani, 2011). The most important ingredient in this mixture is the life-saving antiretroviral drug, efavirenz (Hull, 2010; Rough *et al.*, 2014). This drug shows a profile of CNS effects after administration to patients which could be linked to the possible abuse of this drug (Staszewski *et al.*, 1999; Kryst *et al.*, 2015). Very little proof exist with respect to the abuse potential of this drug, with only one article by Gatch *et al.* (2013) suggesting that the effects of efavirenz are in line with that of LSD, mediated by an agonist effect on the 5-HT_{2A} receptor.

It is known that most drugs of abuse have an effect on the brain reward pathways under the control of DA (Goodman, 2008), although 5-HT (Kirby *et al.*, 2011), NA (Gibbs & Summers, 2002) and EC (Melis *et al.*, 2004) have also been shown to play a crucial role in the mediation of reward, relapse and craving. Furthermore, changes in oxidative stress markers have been

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observed after the administration of various drugs of abuse (Cunha-Oliveira et al., 2008). Not only do drugs of abuse alter brain neurochemistry, they have direct effects on the behaviour of humans and animals (Bardo & Bevins, 2000; Prut & Belzung, 2003; Der-Avakian & Markou, 2012)). Behavioural tests to assess the rewarding or aversive effects of these drugs are therefore critical in a preclinical setting, one of the most popular test being CPP (Bardo & Bevins, 2000). Similarly, the OFT and SPT have found value in assessing whether or not certain drugs of abuse interact with reward pathways in the brain to promote an indulgence in pleasurable activities (Der-Avakian & Markou, 2012).

There are thus various ways to screen for possible rewarding properties of a drug (including neurochemical and behavioural alterations), laying a basis for further in-depth studies.

With recent evidence that dolutegravir, a novel integrase strain inhibitor, also displays similar neuropsychiatric side-effects as efavirenz (Yagura *et al.*, 2017) emphasizes that the addictive-like phenomenon of these drugs present a global risk on health, making it imperative that research be undertaken to uncover the underlying mechanisms of how efavirenz targets the neurocircuitry of reward, its possible addiction biomarkers and how this relates to its abuse potential.

This study will therefore utilize various behavioural studies (including the CPP, OFT and SPT) as well as assess possible alterations in neurochemical and oxidative stress markers to determine whether efavirenz has rewarding properties in animals. This study will add to our understanding of efavirenz abuse, which is reaching alarming proportions in some areas of South Africa.

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Chapter 3

Article

This chapter presents a concept article for submission to *Oxidative Medicine and Cellular Longevity*, an appropriate peer-reviewed scientific journal and prepared according to the authors' guidelines for this specific journal (see Addendum C) or the following link: <https://www.hindawi.com/journals/omcl/guidelines>

The article title page follows which includes the title, contributing authors, the affiliations for each author, the abstract and specific keywords. The structure of the article consists of the following sections: Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Conflict of interest, References.

It should be noted that the format of this concept article has been adapted as to benefit the reader, for this reason, all figures have been inserted in the text. Heading, page and figure numbers in this chapter will align with the dissertation and referral to other chapters will be made for completeness. Before article submission, these changes will be amended

J. Fourie assisted with the design of the study and with the help of M. Möller-Wolmarans conducted the behavioural experiments, undertook the statistical analyses and prepared the first draft of the manuscript. B.H. Harvey also advised on the study design and statistical analysis. M. Möller-Wolmarans and B.H Harvey supervised the study, assisted in the interpretation of the study data, and finalized the manuscript for publication.

All co-authors granted permission for the article to be submitted for the purpose of the MSc.

Title

Evaluation of sub-acute and sub-chronic efavirenz exposure on neurochemical and oxidative stress markers and addictive-like behaviours in rats

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Abstract

News reports indicate the abuse potential of efavirenz in a concoction known as *Nyaope*. This study assessed the possible addictive-like mechanism of efavirenz after sub-acute (5, 10 or 20 mg/kg i.p) and sub-chronic exposure in male Sprague-Dawley rats. The sub-acute study (n = 60) established the most rewarding dose of efavirenz, compared to 1 mg/kg methamphetamine (MA) or vehicle by conditioned place preference (CPP). The sub-chronic study (n = 24) then compared this dosage to a vehicle control w.r.t CPP, sucrose preference, locomotor activity, regional brain monoamines, lipid peroxidation and peripheral superoxide dismutase (SOD). MA and efavirenz at 5 mg/kg induced a similar rewarding effect, while efavirenz at 20 mg/kg induced aversive behaviour compared to the vehicle control in the CPP test. Sub-chronic efavirenz induced: no changes in locomotion and sucrose preference; a significant increase in regional brain lipid peroxidation and peripheral SOD levels; increased frontal cortical dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and serotonin (5-HT) and decreased 5-hydroxyindoleacetic acid (5-HIAA); increased striatal DA, 5-HT and noradrenaline (NA) and decreased DOPAC; as well as decreased hippocampal DA, DOPAC and 5-HIAA. Efavirenz demonstrated significant dose dependant rewarding properties, regional brain monoamine alterations and oxidative stress, highlighting its abuse potential.

Keywords: efavirenz; nyaope; whoonga conditioned place preference; drug abuse; monoamines

3.1 Introduction

More than 56% of people living with human immunodeficiency virus (HIV) receive antiretroviral therapy (ART) which consists primarily of two nucleoside reverse transcriptase inhibitors (NRTI's) such as zidovudine and lamivudine and one non-nucleoside reverse transcriptase inhibitor (NNRTI) of which efavirenz ((4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one) is the most popular [1]. Patients being treated with efavirenz have been described as experiencing insomnia, dizziness, somnolence, vivid and abnormal dreams and thoughts and manic episodes [1-3], whilst some patients describe the feeling as euphoric and dissociative. This profile of side-effects clearly indicates that efavirenz has central nervous system (CNS) activity, which has been ascribed to the lipophilic nature of this compound [4]. Moreover, news reports in South Africa report people crushing and smoking or injecting a white powder, identified as containing efavirenz in combination with marijuana in a mixture commonly known as "Nyaope" or "Whoonga" [5-7]. Nyaope is known to contain rat poison, cocaine and milk powder [8], but the most important constituent in this mixture is efavirenz [7, 9]. The recreational use and abuse of efavirenz places tremendous pressure on the department of health and endangers the lives of patients exposed to it, with reports stating that people are being robbed of their medication which is then sold on the black market [6]. Furthermore, Yagura, Watanabe, Nakauchi, Tomishima, Kasai and Nishida [10] recently highlighted that dolutegravir (an integrase strain inhibitor), also presents with a similar neuropsychiatric side-effect profile than efavirenz. This emphasizes the need to identify possible mechanisms that might relate to the rewarding effects and abuse potential of these drugs. This study will focus specifically on efavirenz.

A recent preclinical study by Gatch *et al.*[11] indicated that efavirenz acts as a weak partial agonist at the serotonin-2A (5-HT_{2A}) receptor, suggesting lysergic acid diethylamide (LSD)-like effects. Additionally, the study included data suggesting interaction with dopamine (DA) and 5-HT transporters, in line with that of other drugs of abuse [11]. In the same study, animals exposed to efavirenz presented with significant decreases in exploratory behaviour in the open field test (OFT) at doses between 10 and 30 mg/kg intra-peritoneally (i.p) and failed to produce self-administration or a preference for efavirenz in a conditioned place preference (CPP) test. Another preclinical study assessed the effects of acute and sub-chronic efavirenz at 25 and 50 mg/kg on neurochemistry, depressive-like behaviour in the forced swimming test (FST) and anxiety-like behaviour in the elevated plus maze (EPM) [12]. Their findings suggest that efavirenz induces anxiety-like behaviour after both acute and sub-chronic exposure to both dosages. However, only sub-chronic dosages at 50 mg/kg induced depressive-like behaviour [12]. Neurochemical analysis revealed that acute doses (25mg and 50mg/kg) of efavirenz

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increased DA, 5-HT and noradrenaline (NA) in the striatum whilst sub-chronic exposure had a contrasting effect in decreasing striatal levels of these monoamines [12]. The findings of both studies [11, 12] indicate dose related effects of efavirenz, suggesting a promising avenue for future studies in this regard.

It is hypothesized that all addictive disorders involve reward processes under the control of one common pathway [13], the mesocorticolimbic reward system where DAergic signalling is of importance in reinforcement [14]. Drugs of abuse have been shown to increase the levels of DA [15-17]. However, 5-HT and NA are also implicated, since the major classes of drugs of abuse increase the levels of these two monoamines in the frontal cortex, striatum and hippocampus, where they induce euphoria, arousal, reward and relapse in humans [18, 19].

Moreover, drugs of abuse (such as cocaine and methamphetamine (MA)) have been shown to increase oxidative stress, being an important process linking alterations in neurocircuitry to the development of neuropsychiatric diseases such as addiction [20]. Oxidative stress is mainly observed in DAergic areas of the brain where the metabolites of DA (3,4-dihydroxyphenylacetic acid (DOPAC), hydrogen peroxide (H₂O₂) and the superoxide anion (O₂⁻)) lead to increased production of reactive oxygen species (ROS) such as quinones, leading to lipid peroxidation and apoptosis, especially if the endogenous antioxidant system (such as superoxide dismutase (SOD)) of the body are compromised [20-22]. Moreover, recent studies have indicated that higher glutamate levels are directly associated with augmented levels of ROS, lipid peroxidation and apoptosis [23, 24], while Cavalcante *et al.*[12] have reported a significant increase in glutamate levels after sub-chronic exposure to both 25mg/kg and 50 mg/kg efavirenz, indicating a possible mechanism through which efavirenz may increase ROS levels.

On the other hand, it should be mentioned that drug abuse does not only alter the neurochemistry of the abuser but also the behaviour. Therefore various animal behavioural tests can be of great importance in screening for the abuse potential of certain drugs [25], with one of the most popular being the CPP test [26]. Through means of classical conditioning, the motivational or aversive properties of a drug serves as an unconditioned stimulus that is repeatedly paired with a neutral environmental stimulus which ultimately leads to the previously neutral environmental stimulus being associated with the drug, thus acting as a conditioned stimulus [26, 27]. In the OFT, alterations in locomotor activity have been observed after administration of drugs of abuse, so that testing for locomotor hyperactivity may be a valuable screening test to assess for rewarding properties [28-32]. Another behavioural test, although not strictly a test to measure addictive-like behaviour, is the sucrose preference test

(SPT). This test provides an indication of whether certain drugs of abuse interact with reward pathways in the brain to promote hedonic activity, i.e. an indulgence in pleasurable activities [33]. DA is known to be directly involved in the motivation to acquire certain rewards while an increase in sucrose consumption has been linked to the ability of animals to experience pleasure [25].

This study aimed to assess the rewarding and addictive-like properties of efavirenz by establishing the most rewarding dosage in a sub-acute study, utilizing the CPP test, in comparison with a well-known drug of abuse, MA, as a positive control. Furthermore, this study aimed to assess any possible alterations in behaviour (in the CPP, OFT and SPT), changes in neurochemistry related to brain reward pathways (viz. frontal cortical, striatal and hippocampal DA, 5-HT and related metabolites, NA) as well as changes in lipid peroxidation and peripheral oxidative stress (SOD), after sub-chronic exposure to the most rewarding dosage of efavirenz (as established in the sub-acute study). It is known that chronic exposure to drugs of abuse cause enduring changes in the neurobiology which is reflected in the dysregulation of monoamines and behaviour [13]. The findings will lay a firm foundation for future studies in this regard and add to our understanding of the abuse potential of efavirenz.

3.2 Materials and methods

3.2.1. Statement on ethics

The study and article were conducted and presented according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines as previously described [34]. The animals were handled according to the code of ethics in research, training and testing of drugs in South Africa, and ethical approval for this study was obtained (NWU - 00267- 16- S5) from the North-West University animal research ethics committee (AnimCare) (NHREC reg. number AREC-130913-015) before commencing with the study.

3.2.1 Animals

Male, adult Sprague-Dawley (SD) rats (200g -250g, Vivarium, North West University) were randomized into 12 rats per exposure group with a total of 7 groups, thus a total of 84 rats were used. The rats were housed under identical conditions in the vivarium (SAVC reg. no. FR15/13458; SANAS GLP compliance no.G0019): cages (230(h) x 380(w) x 380(1) mm) with corncob [35], temperature (21 ± 2 ° C), humidity ($50 \pm 10\%$), white light (350-400 lux), 12 h light/dark cycle and free access to food and water.

3.2.2 Study design

This study consisted of two cohorts, a sub-acute and sub-chronic study. The sub-acute study consisted of five exposure groups receiving either MA (1 mg/kg), efavirenz (at three different

dosages, viz. 5, 10 and 20 mg/kg) or vehicle. The sub-chronic study consisted of two groups receiving either efavirenz (at the most rewarding dosage as determined in the sub-acute study) or vehicle (see study layout in Chapter 1). All animals were randomly assigned to a specific drug exposure group, making use of a block randomization method as published previously [36]. Drug exposure lasted for 6 days in the sub-acute study and 14 days in the sub-chronic study. Apart from the CPP test, the sub-chronic study also included the following behavioural analyses: the OFT, performed on day 13 of exposure and the SPT, performed on day 4 and 14 of exposure. All animals were euthanized (via decapitation) 24hrs after the last behavioural test (CPP post-conditioning test) in the sub-chronic study and regional brain tissue as well as trunk blood were collected

3.2.3 Drugs and drug exposure protocol

Vehicle consisting of 2% methylcellulose [11], adjusted to an average physiological pH, was injected intra-peritoneally (i.p) at 9:00 am on the days of vehicle exposure. Efavirenz (either 5, 10 or 20 mg/kg/day i.p) was administered in a 2% methylcellulose solution at 9:00 am on days of drug exposure, following a previously published method [11]. MA was administered as methamphetamine hydrochloride (1 mg/kg, i.p.) (Sigma-Aldrich, Johannesburg, South Africa) dissolved in saline at 9:00 am on days of drug exposure. The dosage of MA was based on a previous study indicating preference for the drug-paired compartment in the CPP test [37]. The chosen route of administration for all drugs (i.p) is based on a previous study [11].

The sub-acute study was performed to establish the most rewarding dosage of efavirenz in the CPP paradigm compared to a known drug of abuse as the positive control, viz. MA, in comparison to a vehicle group. Efavirenz (at three different dosages) was evaluated following sub-acute dosing in the CPP. Animals were exposed to drug (vehicle, MA or efavirenz) on the mornings of day 1, 3 and 5 and vehicle on days 2, 4 and 6 of drug exposure. A control group of animals only received vehicle (2 % methylcellulose) exposure throughout the 6 days of exposure. This specific vehicle does not induce a place preference in the CPP test [11].

The sub-chronic study compared the most rewarding dosage of efavirenz (established in the sub-acute study) to a vehicle control group (2% methylcellulose). The sub-chronic exposure procedure also alternated between an illicit drug on one day followed by vehicle exposure on the next day, as explained in the sub-acute study. However, this lasted for 14 days of drug exposure. Thus, the sub-chronic groups received efavirenz on days 1, 3, 5, 7, 9, 11 and 13 and vehicle on days 2, 4, 6, 8, 10, 12, 14, following a modification of previous protocols [11, 26]. The vehicle control group received vehicle exposure throughout the 14 days of exposure at 9:00 am each day.

3.2.4 Body weight

The body weight of all animals was determined on post-natal day (PND) 21 and again on each day of drug exposure, in order to calculate the dose of the specific drug to be administered and to ensure equal development across all the exposure groups throughout the study period.

3.2.5 Behavioural analyses

3.2.5.1 Conditioned place preference test (CPP)

To assess the rewarding or aversive properties of a drug, the CPP apparatus and methodology used in this study was adapted from previously published articles and validated in our laboratory (see Addendum A for validation of CPP). Briefly, conditioned testing was conducted in a three-compartment apparatus, constructed of plexiglass, containing three compartments separated by guillotine doors. The two large end compartments (24 X 35 cm) was separated by a smaller centre “choice” compartment (15.5 X 19.5 cm), used on the habituation and test days, as explained below. The two outer compartments were visually different and had different textures on the floor: one compartment having black and white striped walls with a wire mesh floor, and the other compartment having black walls with plexiglass flooring. The middle compartment had grey walls and plexiglass flooring. The CPP paradigm consisted of 3 different stages, lasting 8 days in total for both the sub-acute and sub-chronic studies, as follows:

The first stage (day 1) of the CPP paradigm is the habituation phase, where animals are allowed to freely explore all 3 compartments for 20 minutes, which then provides baseline data assessed as the time spent in each compartment. The compartment in which the least time is spent is then assigned “the drug-paired compartment” while the most preferred compartment is assigned “the vehicle-paired compartment” [26]. The habituation stage was performed the day before the beginning of any drug exposure, in both the sub-acute and sub-chronic study.

The second stage is the conditioning phase, which is done after the habituation and baseline data assessment. Here the animals are injected with the test drug and subjected to the drug-paired compartment for 20 minutes on the designated drug exposure days. On vehicle exposure days, animals are subjected to the vehicle-paired compartment for 20 minutes. Only the outer compartments are used during the conditioning period. This stage was performed from day 1-6 and from day 9-14 of drug exposure in the sub- acute and -chronic studies respectively.

The last stage is post-conditioned testing, where the animals are allowed free access to all the compartments for 20 minutes and the time spent in each compartment is assessed. More time spent in the drug-paired compartment (preference) is evident of a rewarding drug while

less time spent in the drug-paired compartment (aversion) is evident of an aversive drug [38]. This stage of the CPP was performed on the day following the last drug exposure in both the sub-acute and sub-chronic studies.

Behaviour was recorded under dim white light (30 lux) with a digital video camera. Behaviour was scored blindly using EthoVision© XT software (Noldus Information Technology, Wageningen, Netherlands) to assess the time spent in each compartment prior and post-conditioning, presented as the difference in time spent in the drug-paired compartment utilising the following formula: *Time spent in drug-paired compartment during post-test (s) - Time spent in the drug-paired compartment during habituation (s)* [39].

3.2.5.2 Open field test (OFT)

The OFT was performed on day 13 of drug exposure in the sub-chronic study, following a previously published protocol [40]. An open field arena (1m²) was illuminated with dim white light (30 lux) and monitored with a digital video camera, whereupon locomotor activity of the animals (m) were scored for 10 min using EthoVision© XT software (Noldus Information Technology, Wageningen, Netherlands). The arena was cleaned with 10% ethanol solution after each test.

3.2.5.3 Sucrose preference test (SPT)

The SPT was performed on days 4 and 14 of drug exposure to assess for any anhedonic manifestations over time in the sub-chronic study (evident in changes in sucrose consumption) as previously described [41]. During this test, rats were presented with a free choice between two bottles for 24 h, one containing 0.8% sucrose solution and the other tap water. To eliminate the effects of side preference when drinking, the bottles were changed after 12 hours. Both bottles were weighed to measure the amount of water and sucrose solution consumed after the 24 h period. The preference for sucrose was calculated from the amount of sucrose solution consumed, expressed as a percentage of the total amount of liquid consumed (adapted from Rygula, Abumaria, Flugge, Fuchs, Ruther and Havemann-Reinecke [41]).

3.2.6 Neurochemical and peripheral analyses

3.2.6.1 Blood collection

Trunk blood was collected in pre-chilled heparin tubes, centrifuged at 20 000 x g at 4 °C for 10 min and the plasma stored at -80°C until the day of analysis [42]. %SOD was analysed in plasma samples.

Superoxide dismutase (SOD)

Analysis of %SOD was performed using an SOD activity assay kit (BioVision™ Superoxide Dismutase activity assay kit, California, USA, catalogue number: K335-100), utilising water-

soluble tetrazolium (WST)-1 to form a dye following the reduction with the superoxide anion. The rate of reduction by sample superoxide is directly related to xanthine oxidase (XO) activity which is inhibited by SOD. The absorbance was read at 450 nm using a Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA). The SOD activity (inhibition rate %) was calculated using the following equation, as directed by the manufacturer's protocol: % SOD activity (inhibition rate %) = [(blank 1 - blank 3) - sample - blank 2] / (blank 1 - blank 3) x 100. (See Addendum B for additional details)

3.2.6.2 *Brain dissection*

Fresh brain tissue was used for macro-dissection of the frontal cortex, striatum and hippocampus on an ice-cold slab. These brain regions were fixed in relation to anatomical landmarks, as previously described [43, 44]. The frontal cortex, striatum and hippocampus were snap frozen in liquid nitrogen and stored at -80°C until the day of analysis. Brain tissue was used for analysis of monoamines and their metabolites, and lipid peroxidation.

Lipid peroxidation

Regional brain lipid peroxidation was assessed using a thiobarbituric acid (TBA) reactive substances assay kit (Parameter™ thiobarbituric acid reactive substances (TBARS)) assay from R&D Systems (Minneapolis, USA; catalogue number KGE013) based on the principle of malondialdehyde (MDA) reacting with TBA in the presence of heat and acid to produce a coloured product able to absorb light, corresponding to the levels of lipid peroxidation. The total amount of MDA was expressed as MDA (µM) and read at 532 nm using a The Bio-Tek FL600 Microplate Fluorescence Reader spectrophotometric microplate reader, as directed by the manufacturer's protocol.

Regional brain monoamines

Quantification of frontal cortical, striatal and hippocampal DA, 5-HT and respective metabolites (DOPAC and 5-hydroxyindoleacetic acid (5-HIAA)) as well as NA was performed using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), previously validated in our laboratory [45]. The metabolite of NA, 3-methoxy-4-hydroxyphenylglycol (MHPG) was below the limit of detection and therefore not quantified. Sample monoamine concentrations were determined by comparing the area under the peak to that of the internal standard (isoprenaline). All monoamine concentrations are expressed as ng/mg wet weight of brain tissue [35]. The DA and 5-HT turnover ratios were calculated as DOPAC/DA and 5-HIAA/5-HT, respectively [46].

3.2.7 Statistical analyses

Graphpad Prism version 7 for windows (Graphpad software, San Diego, USA) and SAS/STAT® Software were used for the statistical analysis and graphical presentations. All statistical analyses were performed under the guidance of the Statistical Consultation Service of the North-West University. Histograms, Q-Q plots and the Shapiro Wilk test were used to test for normality of all the data sets. Animal body weight (mean \pm SEM) was analysed by two-way analysis of variance (ANOVA) with repeated measures for different days of weight measuring followed by Bonferroni post-hoc analyses. To compare three or more exposure groups as performed in the sub-acute study, one-way ANOVA was used followed by appropriate post-hoc testing using Dunnett's multiple comparison. The nonparametric Kruskal-Wallis test was used if the assumptions of ANOVA were violated. Unpaired student's t-test was used to compare the sub-chronic efavirenz exposure groups to the vehicle exposure group for all behavioural, neurochemical and peripheral blood analysis. Two-way ANOVA with repeated measures for different sucrose preference days (days 4 and 14) followed by Bonferroni post-hoc analyses. The nonparametric Wilcoxon signed-rank test was used if the assumptions of the dependent t-test were violated. In all cases data are expressed as the mean \pm standard error of the mean (SEM), with a p value of <0.05 deemed statistically significant.

3.3 Results

3.3.1 Body weight

All the exposure groups indicated significant and equal growth over the sub-acute and sub-chronic study periods with no significant group differences observed with respect to drug (efavirenz or MA) or vehicle exposure (data not shown).

3.3.2 Behavioural analysis:

Conditioned place preference (CPP)

A one-way ANOVA revealed a significant main effect of drug exposure in the CPP test ($F(4,55) = 13,54$, $p = 0.0001$). Dunnett's post-hoc multiple comparison revealed that animals exposed to sub-acute MA (1 mg/kg) and efavirenz at 5 mg/kg presented with a significant increase in the difference in time spent in the drug-paired compartment compared to a vehicle control group ($p = 0.0009$ and 0.0308 , respectively) (Figure 3-1 A). Animals exposed to sub-acute 10 mg/kg efavirenz displayed no difference in time spent in the drug-paired compartment compared to the vehicle control group ($p = 0.398$) whilst animals exposed to sub-acute 20

mg/kg efavirenz showed a significant decrease in time spent in the drug-paired compartment compared to the vehicle control group ($p = 0.0259$) (Figure 3-1 A).

A significant increase in the time spent in the drug-paired compartment was also observed in animals sub-chronically exposed to 5 mg/kg efavirenz compared to the vehicle control group ($p < 0.01$) (Figure 3-1 B).

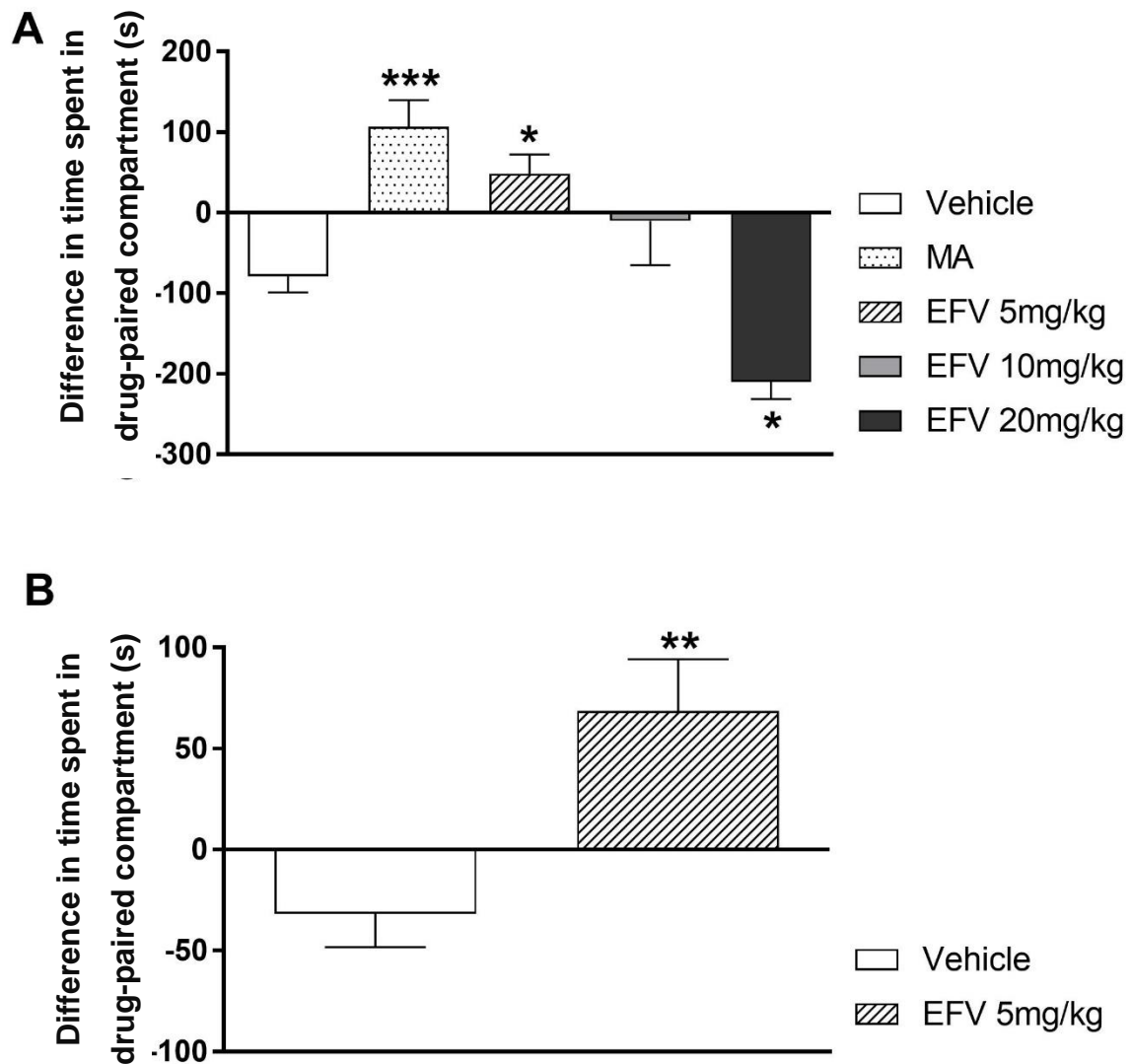


Figure 3-1: Conditioned place preference (CPP) test in A) the sub-acute and B) the sub-chronic exposure groups, receiving the indicated drugs or vehicle. Data presented as difference in time spent in the drug-paired compartment, calculated as described in the Methods. MA, Methamphetamine; EFV, efavirenz. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Vehicle ((A) One-way ANOVA, Dunnett's post-hoc test and (B) Unpaired student's t-test).

Open field test (OFT)

An unpaired student's t-test revealed no significant difference in the total distance travelled between the animals exposed to sub-chronic efavirenz and the vehicle control group ($p = 0.267$) (Figure 3-2).

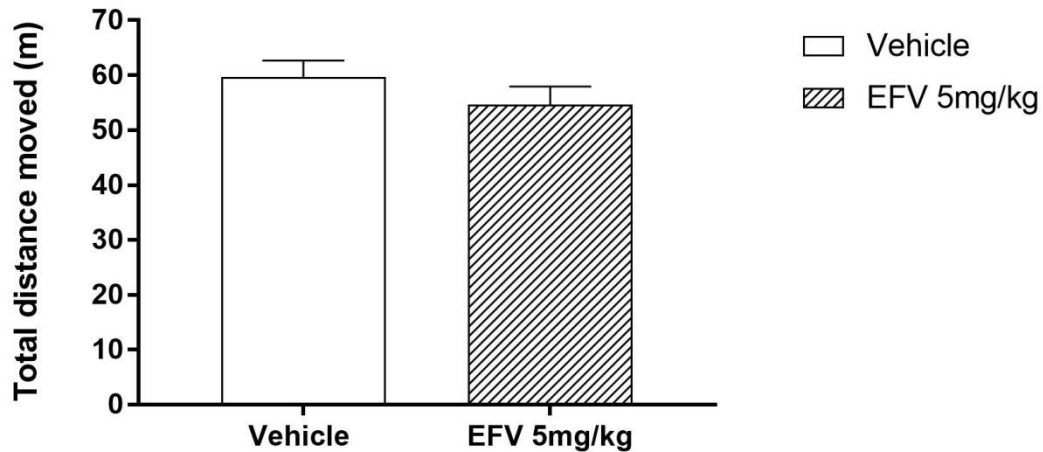


Figure 3-2: Locomotor activity in the sub-chronic study, with rats exposed to vehicle and efavirenz (EFV) 5 mg/kg, respectively (Unpaired student's t-test).

Sucrose preference test (SPT)

A two way ANOVA with repeated measures indicated no significant main effect of efavirenz exposure ($F(1, 11) = 2.826$, $p = 0.12$) or days of sucrose preference ($F(1, 11) = 0.55$, $p = 0.47$). Bonferroni post hoc test also indicated no significant difference in the percentage sucrose consumed between the sub-chronic efavirenz exposed groups and the vehicle control group on either day 4 or 14 (overall $p > 0.99$). There was also no significant difference in the percentage sucrose consumed between day 4 and 14 in the vehicle control and efavirenz groups (overall $p > 0.99$), respectively (Figure 3-3).

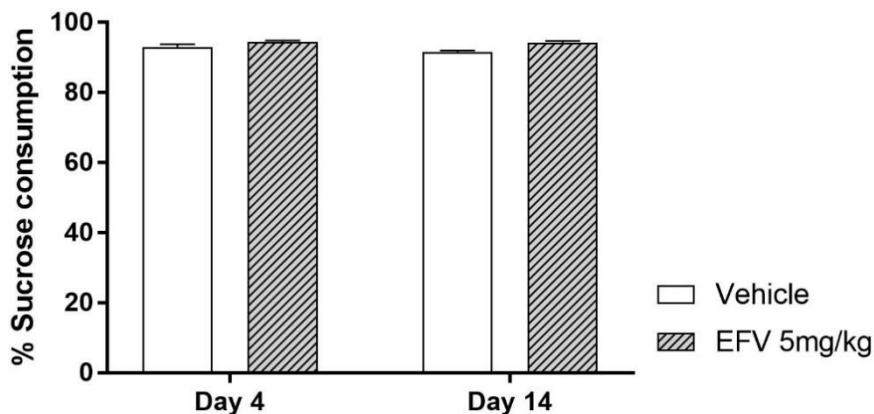


Figure 3-3: %Sucrose consumption in rats exposed to sub-chronic vehicle and efavirenz (EFV) 5 mg/kg, respectively, on day 4 and 14 of drug exposure (Two-way ANOVA with repeated measures and Bonferroni post-hoc test).

3.3.3 Neurochemical analysis:

Regional brain monoamines

All the regional brain monoamine levels obtained in the sub-chronic study are indicated in Table 3-1. An unpaired student's t-test revealed a significant increase in frontal cortical DA ($p = 0.0053$) and DOPAC levels ($p = 0.0453$) in the efavirenz exposed animals compared to the vehicle control group. DA turnover (DOPAC/DA) was significantly decreased in efavirenz exposed animals ($p = 0.0152$) compared to the control group. Frontal cortical 5-HT ($p = 0.0008$) was significantly increased, whilst 5-HIAA levels and 5-HT turnover (5-HIAA/5-HT) were significantly decreased ($p < 0.0001$), in efavirenz exposed animals compared to the vehicle control group. No significant differences were observed between the two exposure groups with regards to frontal cortical NA levels ($p = 0.6460$).

Compared to the vehicle exposed group of animals, the animals exposed to efavirenz presented with a significant increase in striatal DA ($p = 0.0050$), as well as a significant decrease in striatal DOPAC ($p = 0.0403$) and DA turnover (DOPAC/DA) ($p = 0.0440$). Striatal 5-HT was significantly increased ($p = 0.0034$) whilst 5-HIAA ($p = 0.3180$) and 5-HT turnover (5-HIAA/5-HT) ($p = 0.2340$) were unaltered in the efavirenz exposed animals, compared to the vehicle control. Striatal NA was significantly increased in the efavirenz exposed animals compared to the vehicle control ($p = 0.0040$).

Hippocampal DA ($p = 0.0163$) and DOPAC ($p = 0.0060$) were significantly decreased, whilst DA turnover (DOPAC/DA) ($p = 0.0480$) was significantly increased, in the efavirenz exposed animals compared to the vehicle control. Animals exposed to efavirenz had no significant alterations in hippocampal 5-HT ($p = 0.4090$), although 5-HT turnover (5-HIAA/5-HT) ($p = 0.0340$) was significantly reduced and 5-HIAA levels were significantly decreased

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($p = 0.0070$) compared to the vehicle control, while no significant differences were evident for NA ($p = 0.2980$).

Table 3-1: Selected monoamine levels (ng/mg tissue) in regional brain tissue of rats exposed to sub-chronic vehicle (n = 12) or efavirenz (5 mg/kg) (n = 12) respectively. Presented as Mean \pm standard error of the mean (SEM), Unpaired student's t-test.

	Vehicle	Efavirenz (5mg/kg)	<i>p</i> - value
<u>Frontal cortex</u>			
DA	60.7 \pm 27.54	301.2 \pm 72.57	0.0053
DOPAC	40.63 \pm 8.733	149.4 \pm 50.51	0.0453
DOPAC/DA	1.661 \pm 0.3234	0.660 \pm 0.1998	0.0152
5-HT	191.6 \pm 5.873	235.8 \pm 9.696	0.0008
5-HIAA	201.1 \pm 11.64	121.2 \pm 4.637	<0.0001
5-HIAA/5-HT	1.067 \pm 0.080	0.607 \pm 0.666	<0.0001
NA	227.5 \pm 6.304	220 \pm 14.83	0.6460
<u>Striatum</u>			
DA	2228 \pm 238.7	3107 \pm 158.4	0.0050
DOPAC	1109 \pm 162.4	731 \pm 61.48	0.0403
DOPAC/DA	0.626 \pm 0.174	0.249 \pm 0.029	0.0440
5-HT	256.8 \pm 18.7	366.8 \pm 28.02	0.0034
5-HIAA	243.5 \pm 13.42	260 \pm 8.978	0.3180
5-HIAA/5-HT	1.027 \pm 0.131	0.7991 \pm 0.1325	0.2340
NA	81.95 \pm 6.985	137.6 \pm 15.99	0.0040
<u>Hippocampus</u>			
DA	794.8 \pm 223.7	189.5 \pm 63.72	0.0163
DOPAC	384.5 \pm 69.62	152 \pm 31.84	0.0060
DOPAC/DA	0.823 \pm 0.197	1.93 \pm 0.490	0.0480

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5-HT	234.2+30.91	264.6+18.71	0.4090
5-HIAA	250.2+13.26	205.2+7.501	0.0070
5-HIAA/5-HT	1.323+0.227	0.803+0.042	0.0340
NA	279.9+26.74	345.9+55.9	0.2980

DA, Dopamine; DOPAC, 3,4-Dihydroxyphenylacetic acid (DOPAC); 5-HT, Serotonin; 5-Hydroxyindoleacetic acid (5-HIAA), NA, Noradrenaline.

Regional brain lipid peroxidation

An unpaired student's t-test revealed that animals exposed to sub-chronic efavirenz had a significant increase in lipid peroxidation in the frontal cortex and the striatum compared to the vehicle control group ($p = 0.0491$ and $p = 0.0301$, respectively) (Figure 3-4 A and B respectively). However, no significant differences were observed between the sub-chronic efavirenz and vehicle exposed groups with regards to hippocampal lipid peroxidation ($p = 0.114$) (Figure 3-4 C).

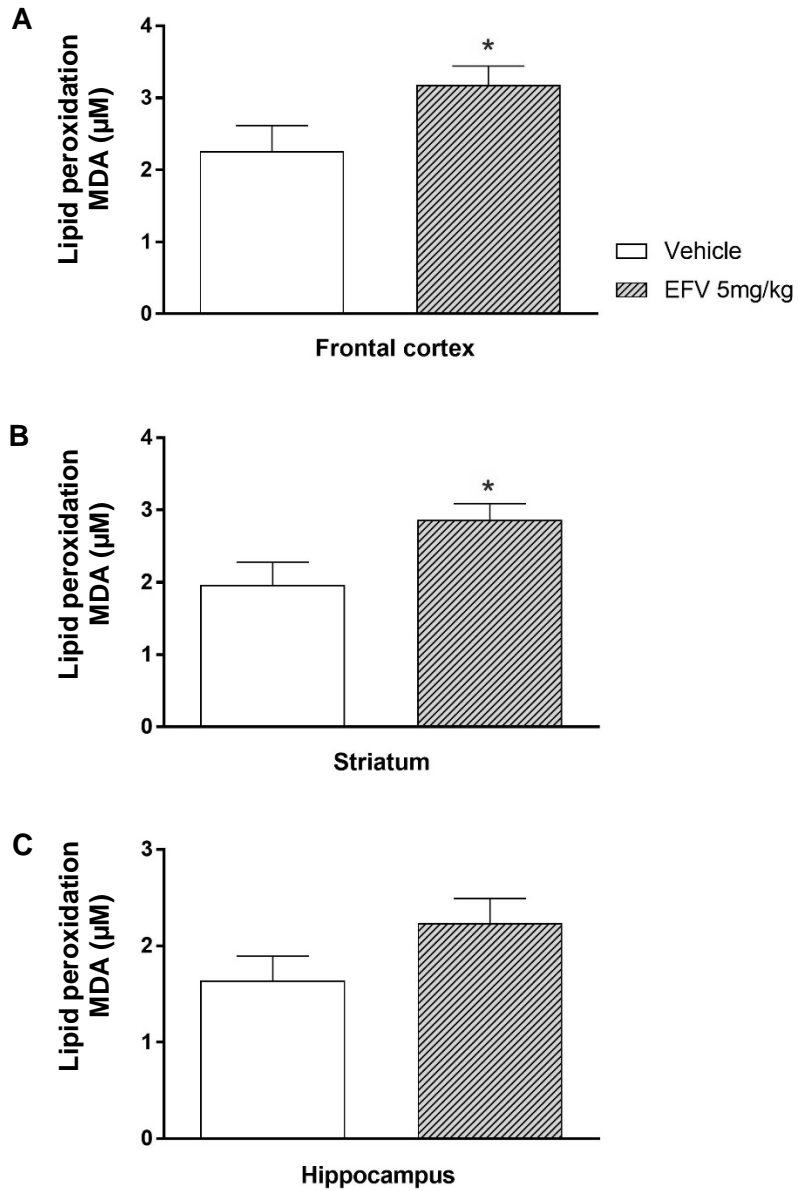


Figure 3-4: Lipid peroxidation levels expressed as MDA (μM) in A) the frontal cortex, B) the striatum and C) the hippocampus in the sub-chronic exposure groups receiving either efavirenz (EFV) 5 mg/kg or vehicle. * $p < 0.05$ vs. Vehicle (Unpaired Student's t-test).

3.3.4 Peripheral analysis:

Percentage plasma superoxide dismutase activity (%SOD)

An unpaired student t-test revealed a significant increase in the %SOD in the sub-chronic efavirenz exposed group compared to the vehicle control ($p = 0.0053$) (Figure 3-5).

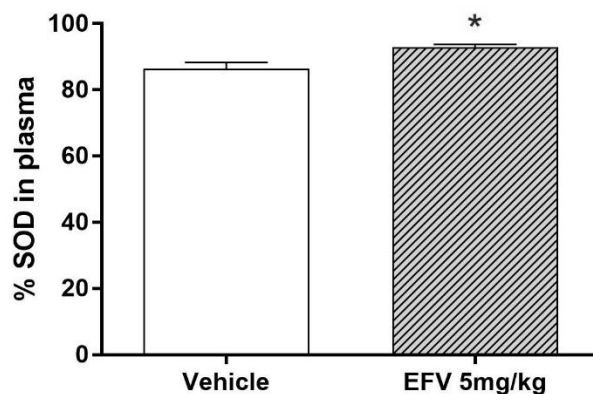


Figure. 3-5: Plasma superoxide dismutase activity (%SOD) in animals exposed to sub-chronic efavirenz (EFV) 5 mg/kg or vehicle. * $p < 0.005$ vs. Vehicle (Unpaired Student's t-test).

3.4 Discussion

The key findings of this study include the observation that animals exposed to sub-acute efavirenz at a dosage of 5 mg/kg spend significantly more time in the drug-paired compartment in the CPP after conditioning, similar to animals exposed to sub-acute MA (1 mg/kg), a known drug of abuse. Moreover, higher doses produce no place preference or are frankly aversive. The 5 mg/kg dose of efavirenz was found to also induce place preference after sub-chronic exposure, while said exposure was accompanied by regional alterations in DA, 5-HT and related metabolites as well as NA and an increase in oxidative stress markers.

Considering the sub-acute study findings, efavirenz was found to induce significant rewarding effects at a very specific dosage, i.e. 5 mg/kg, and to a similar degree as MA, which is a highly addictive and rewarding drug of abuse [37]. Indeed, MA has been found to increase the time spent in the drug-paired compartment after conditioning in the CPP. However, sub-acute efavirenz at 10 mg/kg had no significant effect on the time spent in the drug-paired compartment while a higher dosage (20 mg/kg) induced a significant aversion for the drug-paired compartment, indicating that efavirenz delivers a dose-dependent transition from rewarding to aversive effects. This, however, is true for many drugs of abuse where dosage plays an imperative role with respect to rewarding vs. aversive effects. Delta-9-tetrahydrocannabinol (Δ^9 -THC) (the active ingredient in marijuana) has been shown to only produce a place preference in the CPP after administration of very low dosages (in the range of 0.075 and 0.75 mg/kg) [17, 47], while a higher dosage (6 mg/kg) is aversive [47]. The same is observed after administration of LSD; a lower dosage (0.2 mg/kg) is more rewarding, than higher doses (> 0.2 mg/kg) in the CPP [11, 48, 49]. It is thus evident that some drugs of abuse

(such as LSD and Δ^9 -THC) are rewarding and can cause euphoria and arousal at lower dosages but become aversive at higher dosages due to other side-effects such as dysphoria, anxiety and sedation [17, 47, 48, 50, 51]. The sub-acute CPP results obtained in this study therefore indicate that efavirenz might have dose-dependent rewarding mechanisms, similar to LSD and Δ^9 -THC. This finding essentially validates an efavirenz dose of 5 mg/kg for application in a sub-chronic treatment paradigm.

The rewarding effect of 5 mg/kg efavirenz was duly observed following sub-chronic exposure (14 days), indicating that persistent efavirenz exposure induces sustained CPP over time (no difference observed between sub-acute and sub-chronic CPP- data not shown), signifying long-lasting neuro-adaptive changes that reinforce drug-seeking behaviour. This is in line with other studies, indicating that drugs of abuse induce long-lasting behavioural changes analogous with addiction over time [52-54]. A previous study by Gatch *et al.*[11] indicated that efavirenz failed to produce a CPP after exposure, although methodological differences could explain these discrepancies. The Gatch study exposed animals to either 5 mg/kg or escalating dosages of 10 mg/kg for the first two CPP conditioning sessions and 20 mg/kg for the last two CPP conditioning sessions, which equates to a total of four conditioning sessions. It is possible that 5 mg/kg may have been rewarding, as noted in the current study, but that the various differences in methodology may have altered the outcome. Moreover, they followed an unbiased study design while using a two-compartment apparatus and the visual and textural differences in the two compartments differed from ours, being grid bars on the one floor with the other being a sheet with holes [11]. Moreover, the authors also noted that: *“These outcomes do not rule out the possibility that efavirenz has reinforcing effects under other conditions”* [11].

Interestingly, our study observed no changes in locomotor activity in the animals exposed to sub-chronic efavirenz (5 mg/kg), compared to a vehicle control. Although the psychomotor stimulant theory of drugs of abuse suggests that most drugs of abuse alter the locomotion of animals (reviewed in Weinshenker and Schroeder [30]), we suggest that efavirenz mediates its addictive-like effects through different mechanisms.

When considering neurochemical changes following sub-chronic efavirenz exposure, NA projections in the hippocampus and frontal cortex has been shown to play a crucial role in mediating the alterations in locomotor activity [29], while sub-chronic efavirenz (5 mg/kg) induced a significant increase in striatal NA. That efavirenz induced no significant differences in frontal cortical or hippocampal NA levels, compared to the vehicle control group, and as it did not affect locomotor activity, suggests that striatal NA changes specifically in the ventral striatum implicated in reward related function (eg. nucleus accumbens), as opposed to the

greater basal ganglia regions of the striatum implicated in locomotor function, may underlie the addictive nature of efavirenz. Unfortunately, we were only able to assay total striatal tissue so that the aforementioned hypothesis remains untested until further study. In fact, these findings are in line with a recent study [12] indicating unaltered locomotion in animals exposed to acute or sub-chronic efavirenz, although Cavalcante and colleagues also used different dosages as compared to our study (25 and 50 mg/kg). Moreover, since some animal behavioural analysis protocols require that tests such as the FST (a well-known screening test for depressive-like behaviour in animals) be preceded by the OFT to ensure that a false positive or negative is not due to immobility [55], the unaltered locomotor activity observed in the OFT may in this instance be of great importance in supporting the CPP results, indicating that a side preference was not due to immobility. Since a previous preclinical study suggests that sub-acute efavirenz at 25 or 50 mg/kg does not alter the spontaneous locomotion of animals [12], OFT was only assessed in the sub-chronic efavirenz exposure groups in the current study.

Although the SPT is not strictly a test to assess for addiction or reward, many studies have utilised this simple test to screen for the possibility that a drug may interact with the reward pathways of the brain [33], while it has become especially useful in assessing anhedonia as a co-presenting symptom of depression, see for e.g. Der-Avakian & Markou [33]. Previous clinical studies have indeed observed that efavirenz is known to induce psychological side-effects such as anhedonia and depression [56-58]. However, in this instance, sub-chronic efavirenz at 5 mg/kg failed to evoke any difference in sucrose consumption compared to a vehicle control group on either day 4 or 14 of drug exposure, indicating no hedonic manifestations in a time related manner. Hedonia and anhedonia are closely linked to increased or decreased DA function, respectively, in the limbic reward pathways [33, 41, 59]. Thus, a decrease in DA levels in the frontal cortex have been directly linked to anhedonia and a decrease in experiencing pleasure [33, 60, 61]. However, the current study observed an increase in frontal cortical DA and DOPAC after sub-chronic efavirenz (5 mg/kg) exposure, as well as an increase in striatal DA, which would explain why sucrose consumption was unaltered in these animals. Thus, although further study is needed, it would appear that efavirenz does at least *maintain* hedonic behaviour in keeping with an overall beneficial effect on DA reward pathways. Unfortunately, the sub-chronic study did not investigate higher doses of efavirenz (e.g. 20 mg/kg) that had displayed aversive behaviours in the CPP following sub-acute treatment and that would be expected to be dysphoric following prolonged exposure. Interestingly though, a previous study suggests that a 50 mg/kg as compared to 25mg/kg dose of efavirenz induced an *increase* in depressive-like behaviour [12], thus supportive of the outcome that lower dosages of efavirenz are rewarding via a bolstering of DA reward

pathways, while higher doses are aversive, the latter due to side-effects such as depression and anhedonia.

As mentioned previously it is well-known that drugs of abuse induce alterations in neurochemistry, with DA playing a crucial role in mediating reward and learning [13, 62]. Sub-chronic efavirenz at 5 mg/kg significantly increased DA levels in the frontal cortex (the brain region involved in reward and learning [25]) as well as the striatum (involved in decision making and reward learning [63]). Both these regions are rich in D₁ receptors [64], which is associated with the reward of food, drugs and alcohol as well as the reinforcement of drug seeking behaviour, place preference for drugs of abuse and enhancement of palatability of food [61, 65, 66]. The increased DA levels observed in these specific brain regions are therefore in line with the rewarding effects of sub-chronic efavirenz observed in the CPP. Interestingly, sub-chronic efavirenz significantly decreased DA levels in the hippocampus (implicated in memory and reward anticipation); this region contains high densities of D₂ receptors with high affinity for DA [67]. After long-term activation of these presynaptic receptors (functioning as auto receptors), DA release is decreased [61, 68]. Furthermore, constant high levels of DA activate the D₂ receptors in a tonic manner that maintains the motivation to procure a drug [14, 61]. The observed reduction in DA turnover in the frontal cortex and striatum after sub-chronic efavirenz exposure suggests diminished DA metabolism via monoamine oxidase (MAO) [20] and thus an increase in the bioavailability. This is evident in the significantly higher DA levels in these brain regions. The increased DA turnover after sub-chronic efavirenz exposure in the hippocampus indicates an increase in DA metabolism, further elucidating the significantly lower DA levels in this region and in line with previous studies [12, 63].

With regards to 5-HT, numerous studies indicate that known drugs of abuse interfere with 5-HT reuptake and release, and that this system is modulated by all major classes of drugs of abuse [69]. One such group includes the psychostimulants (such as cocaine and MA) which, in addition to interfering with the DA transporters to increase DA, also increase prefrontal 5-HT levels by blocking the 5-HT transporters [19]. After sub-chronic exposure to efavirenz, frontal cortical and striatal 5-HT levels were significantly increased, without any noticeable effects on hippocampal 5-HT, which is in accordance with previous studies on known drugs of abuse [19]. 5-HT is intimately involved in behaviour, emotion and memory processes regarding drug addiction [19, 69-71], while recent studies in rodents have found that efavirenz has noteworthy interactions with both 5-HT and DA transporters [11]. These actions may play a direct role in how efavirenz increases DA and 5-HT levels in the above-mentioned brain regions, as presented in this study after sub-chronic exposure. In this regard, the 5-HT_{1A} and 5-HT_{2A} receptors play an important role in drug addiction: 5-HT_{1A} receptors provide excitatory

input on DA neurons, resulting in an increase in DA release [72], while the latter represent the primary binding site for all the hallucinogens, such as LSD [73]. After binding of the hallucinogens to the 5-HT_{2A} receptor, the increased serotonergic transmission leads to an increase in glutamate release and resulting hyper-glutamatergic transmission in the frontal cortex, inducing downstream effects on DA release in the striatum [74]. Recently, Gatch *et al.*[11] confirmed that efavirenz (30 mg/kg, i.p for 6 days of alternate day dosing) has noteworthy agonistic effects on the 5-HT_{2A} receptor, which they argue is the primary reason for its possible hallucinogenic effects. The current study did not observe any changes in hippocampal 5-HT levels, although this is in line with other studies on drugs of abuse such as amphetamine [75, 76]. The decrease in 5-HT turnover observed in the frontal cortex and hippocampus of animals exposed to sub-chronic efavirenz might imply that the increased levels of 5-HT in these brain regions is due to a decrease in 5-HT metabolism, which is in line with another study where rats were sub-acutely exposed to efavirenz at doses of 25 and 50 mg/kg [12]. However, contrary to an increase in striatal DA, 5-HT and NA described in the current study, Cavalcante *et al.*[12], observed that chronic exposure to efavirenz reduced all striatal monoamines, viz. DA, 5-HT and NA. However, they did not describe monoaminergic effects in the hippocampus and frontal cortex which would have provided for interesting comparison. Yet, the latter findings were observed after the administration of doses ranging from 25 mg/kg to 50 mg/kg, much higher than the dosage used in the current study. These doses coincide with the aversion noted for 20 mg/kg efavirenz described in the CPP test after acute treatment. The reduction in striatal monoamines following 25 mg/kg to 50 mg/kg efavirenz, as observed by Cavalcante *et al.*[12] therefore correlates with depressive and anxiety-like behaviour described in their report, and dovetails with our suggestion that efavirenz produces rewarding effects in animals at lower dosages. Literature also suggests that the neuropsychiatric side effects of efavirenz, in a clinical setting, are directly related to its plasma concentration [2], where higher dosages induces dizziness, vertigo, confusion somnolence, nervousness, irritability and depression [2, 12]. Moreover, the dosages and concentrations of efavirenz have been directly linked to plasma levels of the metabolite, 8-hydroxy-efavirenz which is thought to be responsible for many of the adverse-effects of efavirenz [77, 78]. In fact, 8-hydroxy-efavirenz is a pro-oxidant that is believed to be directly responsible for inducing redox dysregulation in patients treated with efavirenz [77, 79]. Moreover, altered redox is well-described in both mood disorders and psychosis [80] so that it is well worth investigating whether the above-mentioned effects of efavirenz are associated with oxidative stress.

Apart from the neurochemical alterations induced by drugs of abuse, neurotoxicity is another important factor that might explain some of their neuropsychological effects [81]. The mechanism whereby DA induces neurotoxicity resides in the formation of free radicals, O₂⁻,

H₂O₂ and OH as well as quinone adducts [20] especially in DA rich regions of the brain [20, 82] SOD is responsible for scavenging these free radicals and protecting the body against oxidative stress which would otherwise lead to lipid peroxidation and cell death [20]. Indeed, our study observed that animals exposed to sub-chronic efavirenz had significantly increased plasma SOD activity. Moreover, significantly elevated lipid peroxidation was evident in the striatum and frontal cortex, but not the hippocampus in animals exposed to sub-chronic efavirenz, which further supports the presence of oxidative stress and in particular that redox disequilibrium originates in DA rich areas of the brain. Thus, an increase in ROS and free radicals caused by augmented DA levels prompted an increase in SOD activity in an attempt to detoxify these free radicals. These findings are precisely in line with preclinical studies evaluating the effects of cocaine on SOD and lipid peroxidation in the frontal cortex and striatum of rats [83]. Moreover, it should be noted that efavirenz not only increases oxidative stress, but also increases endoplasmic reticulum stress and autophagy in human endothelial cells [84]. It can therefore be argued that the observed elevated lipid peroxidation is due to the cytotoxic nature of efavirenz or 8-OH-efavirenz or both. However, the increased levels of lipid peroxidation were only observed in the brain regions (frontal cortex and striatum) where DA levels were also significantly increased in line with the above-mentioned hyper-dopaminergic induced oxidative stress explanation. Furthermore, increased oxidative stress and ROS have been linked with an increase in glutamate levels in the brain (specifically glutamate rich areas), observed in various studies [23, 85]. Cavalcante *et al.*[12] reported a significant increase in glutamate levels in the striatum after sub-chronic exposure to efavirenz at doses of 25 mg/kg and 50 mg/kg, possibly providing another explanation for evidence of oxidative stress in sub-chronic efavirenz exposed rats although at much higher dosages than the current investigation.

3.5 Conclusion

We show here for the first time that animals exposed to sub-acute efavirenz present with a preference for the drug-paired compartment in a CPP paradigm, closely paralleling the effects of MA. That this response occurred following exposure to a low (5 mg/kg) but not high dosage of efavirenz is thus confirmatory that the rewarding properties of this compound are dose-related. Furthermore, higher doses of efavirenz are either ineffective in the CPP or frankly aversive. Exposure to sub-chronic efavirenz at 5 mg/kg also displays addictive-like properties in the CPP paradigm, while at the same time presents with concomitant alterations in regional brain neurochemistry, lipid peroxidation and peripheral oxidative stress that are congruent with known drugs of abuse that target DA reward pathways. The results acquired in these sub-acute and sub-chronic studies have significant relevance in elucidating the mechanism through which efavirenz induces certain neuropsychiatric and addictive effects in humans,

extending the evidence base of its reported abuse and may aid in the development of new strategies to diminish these effects and to improve ART. However, it should be taken into account that the rewarding properties of efavirenz observed at 5mg/kg is at lower dose than clinical application. Further research should evaluate the subjective effects in a clinical setting.

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3.7 Conflict of interest

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

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Chapter 4

Summary, conclusions and recommendations

This chapter serves as a summary of the results and discussion as presented in chapter 3, as well as Addendum A. In order to give a comprehensive overview, make a final conclusion and recommendations for future studies, the outcomes and hypothesis will be considered.

4.1 Summary of results

It has been reported that the antiretroviral (ARV) drug, efavirenz, induces various central nervous system (CNS) side effects which include euphoria, manic episodes, somnolence, vivid and abnormal dreams and thoughts and depression (Marzolini *et al.*, 2001; Fumaz *et al.*, 2002; O'Mahony *et al.*, 2005; Kenedi & Goforth, 2011; Apostolova *et al.*, 2015). Recently it has come to light that efavirenz is being used recreationally and abused for its mind altering effects. However, very little evidence of the abuse potential of this drug exists and thus far only a few studies have observed an interaction of efavirenz with certain targets that may confer addictive properties, viz. the 5-HT_{2A} receptor, the serotonin transporter (SERT), dopamine (DA) transporter (DAT) and muscarinic (M) receptors (Gatch *et al.*, 2013; Cavalcante *et al.*, 2017). Drugs of abuse are known to interact with reward pathways under the direct control of DA (Goldstein & Volkow, 2002; Goodman, 2008), although serotonin (5-HT) and noradrenaline (NA) have also been observed to play a crucial role in mediating reward, learning and relapse in humans (Gibbs & Summers, 2002; Müller *et al.*, 2007; Weinshenker & Schroeder, 2007; Ferenczi *et al.*, 2016). Furthermore, numerous behavioural changes are observed after exposure to drugs of abuse (Bardo & Bevins, 2000; Kalivas & McFarland, 2003; Villégier *et al.*, 2003). One of the most popular and valuable preclinical behavioural tests to assess and screen whether a drug possesses rewarding properties is the conditioned place preference (CPP) test (Buccafusco, 2000; Braida *et al.*, 2004; Tzschentke, 2007; Zakharova *et al.*, 2009).

In this study we established that the well-known drug of abuse, methamphetamine (MA) (1 mg/kg/d x alternate day x 6 days), has significant rewarding properties in the CPP, evident as increased time spent in the drug-paired compartment (compared to a vehicle control), thus validating the test for application in our study (Addendum A). MA was subsequently used as the positive control for the study. Thereafter (Chapter 3), we reported that sub-acute efavirenz

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(5 mg/kg/d alternate days x 6 days) induced significant rewarding properties in the CPP test, evident as an increased time spent in the drug-paired compartment (compared to a vehicle control) and comparable to that of the aforementioned dose of MA (see Table 4-1). Higher doses of sub-acute efavirenz were progressively less rewarding, with the highest dose (20 mg/kg) being significantly aversive in the animals. In the latter instance, animals spent significantly less time in the drug paired-compartment after conditioning in the CPP test. Similarly, sub-chronic efavirenz (5 mg/kg/d alternate days x 14 days) induced significant rewarding properties in the CPP test (Chapter 3), thus signifying long-lasting neuro-adaptive changes that reinforce drug seeking behaviour analogous with addiction over time (Do Couto *et al.*, 2005; Valjent *et al.*, 2006). In addition, we also tested additional behavioural parameters that may highlight possible rewarding or addictive-like properties of the drug (Chapter 3), viz. assessment of anhedonia in the sucrose preference test (SPT) (Rygula *et al.*, 2005; Tonissaar *et al.*, 2006) and screening for locomotion alterations in the open field test (OFT) (Villégier *et al.*, 2003; Zakharova *et al.*, 2009). These two behavioural measures provide an indication of whether certain drugs of abuse interact with reward pathways in the brain (Der-Avakian & Markou, 2012; Aarde *et al.*, 2013). However, sub-chronic efavirenz had no noticeable effect in these tests vs. vehicle-treated animals (see Table 4-1). This would suggest that efavirenz does not elicit its rewarding properties through the same mechanism as does many other drugs of abuse and that efavirenz at least maintains hedonic behaviour in keeping with an overall beneficial effects on DA reward pathways.

Given that most drugs of abuse present with demonstrable effects on regional neurochemistry and oxidative stress markers (Gibbs & Summers, 2002; Goldstein & Volkow, 2002; Müller *et al.*, 2007; Goodman, 2008), we also evaluated the effects of sub-chronic efavirenz (5 mg/kg/d alternate days x 6 days) on regional brain DA, 5-HT and their related metabolites as well as NA and cortico-striatal lipid peroxidation (Chapter 3). Efavirenz significantly increased frontal cortical DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-HT levels whilst 5-hydroxyindoleacetic acid (5-HIAA) levels were significantly lower compared to vehicle exposed animals. Frontal cortical DA and 5-HT turnover was significantly decreased with NA unaffected, in animals exposed to sub-chronic efavirenz compared to a vehicle control (see Table 4-1).

Striatal DA, 5-HT and NA were increased whilst DOPAC levels and DA turnover were decreased and 5-HIAA levels and 5-HT turnover were unaffected in animals exposed to sub-chronic efavirenz compared to a vehicle control (see Table 4-1).

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Hippocampal DA, DOPAC, 5-HIAA and 5-HT turnover was significantly decreased whereas DA turnover rate was significantly increased and NA and 5-HT levels unaffected in animals exposed to efavirenz compared to a vehicle control (see Table 4-1).

A significant increase in regional brain lipid peroxidation was noted in the striatum and frontal cortex but not in the hippocampus after sub-chronic exposure to efavirenz, suggesting oxidative stress was more prominent in the DA rich regions of the brain. In addition, we observed significantly higher levels of plasma SOD activity in efavirenz exposed animals compared to a vehicle control.

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Table 4-1: Summary of the bio-behavioural findings of efavirenz in (A) a sub-acute study employing efavirenz at three different dosages (5, 10 and 20 mg/kg//d alternate days x 6 days), compared to methamphetamine (MA; 1mg/kg) and vehicle control, and (B) a sub-chronic study comparing efavirenz (5 mg/kg/d alternate days x 14 days), to vehicle control. Male Sprague Dawley (SD) rats (200-250g), n = 12 per group were used.

(A) Sub-acute study									
		Behavioural analysis							
		CPP test (time in drug-paired compartment)							
Methamphetamine vs. vehicle		↑							
Efavirenz 5 mg/kg vs. vehicle		↑							
Efavirenz 10 mg/kg vs. vehicle		O							
Efavirenz 20 mg/kg vs. vehicle		↓							
(B) Sub-chronic study									
		Behavioural analysis							
Efavirenz 5 mg/kg vs. vehicle		CPP (time in drug-paired compartment)			OFT			SPT	
		↑			O			O	
		Neurochemical analysis							
		DA	DOPAC	DOPAC/DA	5-HT	5-HIAA	5-HIAA/5-HT	NA	LP
	<i>Frontal cortex</i>	↑	↑	↓	↑	↓	↓	O	↑
	<i>Striatum</i>	↑	↓	↓	↑	O	O	↑	↑
	<i>Hippocampus</i>	↓	↓	↑	O	↓	↓	O	O
	Peripheral %SOD activity								
<i>Plasma</i>	↑								

↑: Significant increase; ↓: significant decrease; O: no significant/noticeable differences. Abbreviations: CPP, conditioned place preference test; OFT, open field test; SPT, sucrose preference test; DA, Dopamine; DOPAC, 3,4-Dihydroxyphenylacetic acid; 5-HT, Serotonin; 5-Hydroxyindoleacetic acid, 5-HIAA; NA, Noradrenaline; SOD, superoxide dismutase; LP, lipid peroxidation.

4.2 Study aims and relevant findings

1) To validate the CPP paradigm in our laboratory by using MA as a positive control at a dose of 1 mg/kg (Addendum A)

After animals were conditioned in the CPP paradigm with MA at 1 mg/kg for three sessions, a significant increase in time spent in the drug-paired compartment was observed compared to animals receiving a vehicle control (2% methylcellulose). The current literature clearly indicates that MA possesses rewarding properties (Toussaint, 2017). We thus established and validated a trustworthy paradigm to assess for rewarding properties of drugs in our laboratory utilising a CPP paradigm protocol modified from previous studies (Lepore *et al.*, 1995; Buccafusco, 2000; Tzschentke, 2007; Prast *et al.*, 2014) (see Addendum A for details).

2) Evaluate the possible addictive-like behaviour (assessed in the CPP test) induced by sub-acute efavirenz exposure at three different dosages (viz. 5, 10, 20 mg/kg/d x alternate days x 6 days), in order to determine the most rewarding dosage of efavirenz (Chapter 3).

We observed that animals exposed to sub-acute efavirenz at 5 mg/kg spent significantly more time in the drug-paired compartment after 3 conditioning session in the CPP paradigm, whilst 10 mg/kg induced no significant difference. Sub-acute efavirenz at 20 mg/kg induced a significant aversion for the drug-paired compartment in the CPP paradigm. Since more time spent in the drug-paired compartment after conditioning in the CPP is indicative of a rewarding effect of a drug in animals (Buccafusco, 2000), we conclude that lower, but not higher dosages (20 mg/kg) of efavirenz are significantly rewarding as tested in our CPP paradigm.

3) To investigate the effect of sub-chronic efavirenz administration (dose as determined in the sub-acute study) on locomotor activity (in the OFT), preference for sucrose (in the SPT) and addictive-like behaviour (in the CPP), compared to animals receiving only vehicle (Chapter 3).

Animals sub-chronically exposed to efavirenz at 5 mg/kg efavirenz (determined as the most rewarding dosage in the sub-acute study) presented with an increase in time spent in the drug-paired compartment after 3 conditioning sessions in the CPP, compared to a vehicle control. However, no changes were observed in the OFT (locomotion was unaltered) and in the SPT after sub-chronic exposure to efavirenz at 5mg/kg compared to a vehicle control. The results obtained in the SPT supports the findings that a lower dosage of efavirenz, as used in this study, has rewarding properties with no indication of anhedonic manifestations over time. Studies suggest that some of the adverse neuropsychiatric side-effects of efavirenz only presents at higher dosages (Marzolini *et al.*, 2001; Langmann *et al.*, 2002; Lochet *et al.*, 2003;

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Tovar-y-Romo *et al.*, 2012). However, the current study did not investigate the sub-chronic effects of efavirenz at 20 mg/kg in the SPT which would have provided for interesting comparison.

4) Using the most rewarding dosage of efavirenz (as determined in the sub-acute study), regional brain neurochemistry (viz. DA, 5-HT and related metabolites as well as NA levels), regional brain lipid peroxidation, as well as plasma levels of SOD, were assessed and compared to animals only exposed to a vehicle (Chapter 3).

Various biochemical alterations were observed after animals were exposed to sub-chronic efavirenz at 5 mg/kg. These findings are summarised in Table 4-1 and discussed in detail in Chapter 3. In short, the alterations in DA, 5-HT, NA and related metabolites were in line with previous studies evaluating drugs of abuse, observing similar alterations in neurochemistry and oxidative stress markers (Leshner, 1997; Balleine *et al.*, 2007; Müller *et al.*, 2007; Cunha-Oliveira *et al.*, 2013; Cavalcante *et al.*, 2017). Lipid peroxidation was also significantly increased in the DA rich regions of the brain (frontal cortex and striatum) as well as peripheral SOD levels after sub-chronic exposure to efavirenz at 5 mg/kg. These observed changes in neurochemistry and oxidative stress markers may underlie a possible mechanism through which efavirenz induces many of its CNS effects; efavirenz has noteworthy interaction on the SERT, DAT and MAO-A enzyme which are responsible for the reuptake and metabolism of the monoamines (5-HT, DA and NA) as previously confirmed and discussed in Chapter 2 and 3. An increase in these monoamines is observed after the administration of drugs of abuse, in line with the findings in the current study (see Figure 4-1). These increases in monoamines (as observed in the current study) play a role in reward, learning, palatability, cravings, relapse and arousal associated with drugs of abuse. Increased DA levels are congruent with an increase in ROS, lipid peroxidation and cell death. Furthermore, efavirenz has been shown to interact with the 5-HT_{2A} receptor, leading not only to various behaviour alterations and manifestations such as hallucinations, but also increases DA through hyper-glutamatergic transmission further exacerbating oxidative stress such as increased lipid peroxidation, contributing to the development of neuropsychiatric symptoms. The antioxidant-system of the body (e.g. SOD) is responsible for scavenging the ensuing ROS (see Figure 4-1)

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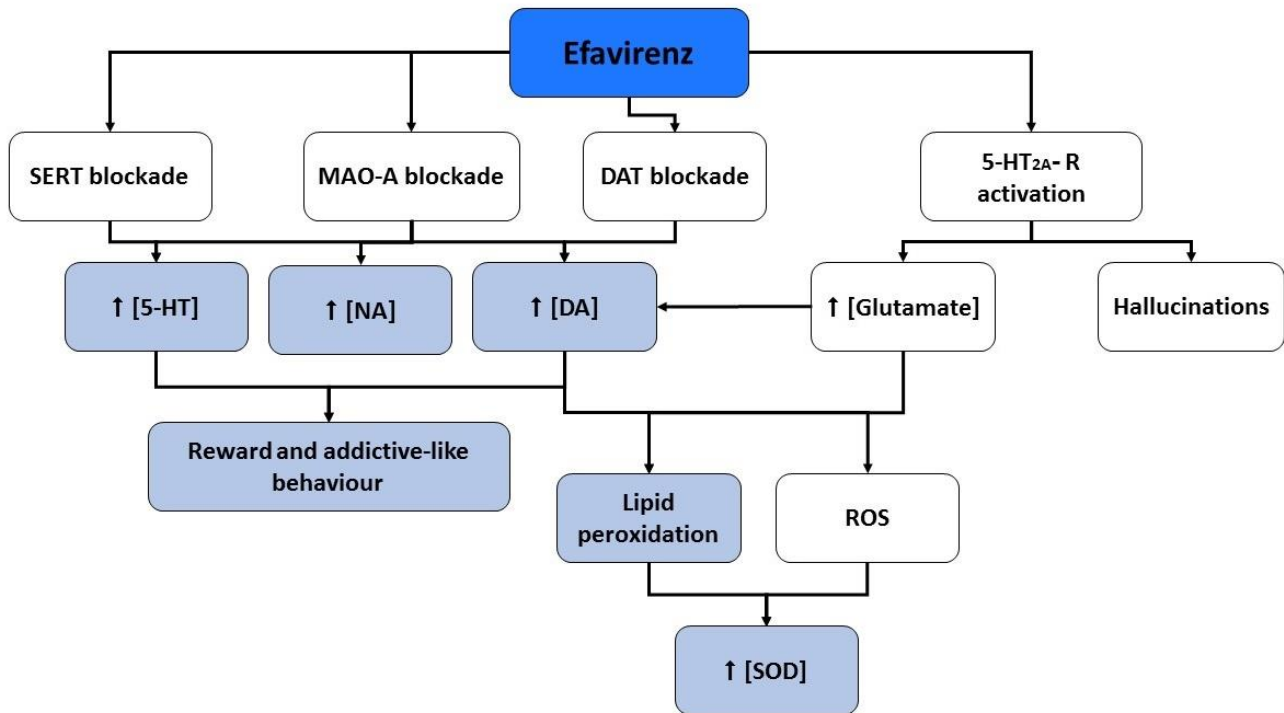


Figure 4-1: Simplified hypothesis concerning the mechanism through which efavirenz elicits its effects, taking into account the findings of the current study as well as findings from previous preclinical and clinical studies. Monoamine alterations are region specific (consolidate Table4-1 for detail). Serotonin transporter (SERT), dopamine transporter (DAT) monoamine oxidase (MAO)-A, serotonin (5-HT), dopamine (DA) noradrenaline (NA), reactive oxygen species (ROS), superoxide dismutase (SOD). Blue squares indicate findings in the current study.

4.3 Recommendations

- Reports describe people smoking efavirenz in combination with other constituents including marijuana (Hull, 2010; Cullinan, 2011; Fihlani, 2011; Rough *et al.*, 2014). Studies have indicated that the active ingredient in marijuana, tetrahydrocannabinol (Δ^9 -THC) has addictive properties (Lepore *et al.*, 1995; Braida *et al.*, 2004). Further research is needed to investigate whether the rewarding effects of efavirenz are amplified or altered when used in combination with marijuana compared to the effects elicited by efavirenz alone.
- The fact that efavirenz is being smoked gives rise to another research problem, being that there is uncertainty as to whether some psychoactive constituents of efavirenz are present in the smoked form alone. Indeed, possible structural changes may occur after heating of the compound, although no studies to date have investigated this question. Efavirenz has a melting point of 139°C-141°C (O'Neil, 2013) and studies show that the average temperature of a lit cigarette varies between 470° and 812° C and can reach

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temperatures as high as 950°C (Baker, 2006) thus, much higher than the melting point of efavirenz. It is therefore possible that structural changes could take place when efavirenz is smoked giving rise to efavirenz derivatives which are possibly psychoactive and may be more rewarding or addictive than the original efavirenz compound. Future research should evaluate the structural changes (if any) elicited by the heating of efavirenz and investigate whether the heated compound possesses any rewarding effects and if those rewarding effects are greater than that of the original compound. It is however possible that smoking efavirenz improves the kinetics and absorption due to the aromatizing of the original compound leading to improved and rapid uptake into the system. Further study into this might bring forth new data and lend great insight into this practice.

4.4 Novel findings and conclusion

In a sub-acute study, utilising MA as a positive control, a trustworthy paradigm (the CPP test) to assess for the rewarding properties of drugs was established. Using this same paradigm, we observed for the first time that efavirenz presents with dose-dependent rewarding properties; animals conditioned with low dosages (5 mg/kg) showed a significant preference for the drug-paired compartment whilst animals exposed to higher dosages (20 mg/kg) showed significant aversion for the drug-paired compartment, with the intermediate dose (10 mg/kg) showing no difference. Furthermore, animals exposed sub-chronically to efavirenz at 5 mg/kg presented with various behavioural and neurochemical alterations compared to a vehicle control. These animals spent significantly more time in the drug-paired compartment in the CPP test, indicating possible chronic abuse potential of this drug. Efavirenz at 5mg/kg sub-chronically however did not induce any signs of anhedonia (as determined in the SPT) or altered locomotor activity. This suggests that a low dose of efavirenz (5mg/kg) does not induce many of the dysphoric effects reported in literature.

In line with other drugs of abuse, sub-chronic efavirenz at 5 mg/kg induced significant alterations in the neurochemistry of the animals compared to a vehicle control, including a range of effects on DA, 5-HT, NA and related metabolites in the frontal cortex, striatum and the hippocampus that may have implications for how efavirenz elicits a rewarding response in users. These changes in neurochemistry are further supported by significant increases in lipid peroxidation in DA rich regions of the brain and a noticeable increase in peripheral plasma SOD activity, which suggests that efavirenz-induced oxidative stress-mediated changes in monoamine content in the reward centres of the brain may drive drug-seeking behaviour. Collectively, the observed alterations in behavioural, neurochemical and oxidative stress markers in this study have succeeded in elucidating possible mechanisms through which

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efavirenz may elicits many of its neuropsychiatric side-effects and sheds light on the reported recreational use and abuse of this drug in humans.

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Addendum A

Validation of the conditioned place preference (CPP) paradigm

The outcome of this study relied on a specific behavioural analysis to evaluate the possible addictive-like properties of efavirenz following both acute and sub-acute exposure (6 days), viz. the conditioned place preference (CPP) test. Thus, before we undertook the study it was imperative that we establish whether we have set up this test correctly in our laboratory and that the test measures what it is intended to measure. This addendum therefore describes the validation of the CPP using a positive control (a well-known drug of abuse) in a sub-acute CPP paradigm, as published previously (Pickens et al., 1978; Tuazon et al., 1992; Zakharova et al., 2009; Logan et al., 2016; Toussaint, 2017).

A1.1 Introduction

The CPP test is one of the most popular and widely used preclinical tests to assess for rewarding properties of drugs (Bardo & Bevins, 2000; Tzschentke, 2007). In this paradigm, classical Pavlovian conditioning is used to assess the motivational or aversive properties of a drug, where these effects of the drug serve as an unconditioned stimulus that is repeatedly paired with a neutral environmental stimulus, which ultimately leads to the previously neutral environmental stimulus being associated with the drug thus acting as a conditioned stimulus (Bardo & Bevins, 2000).

Many variations of the same test exist where differences in design and methodology as presented in the literature may alter the outcome of the results. In this test the basic principles remain the same: the stimulus of choice (e.g. the potentially rewarding or aversive drug) is administered and paired with a distinctive compartment, these compartments vary in visual and textural aspects to ensure a distinctive character between the drug and vehicle paired compartments. The compartment should vary in flooring texture, colour or colour pattern and olfactory cues (Bardo & Bevins, 2000; Buccafusco, 2000; Zakharova *et al.*, 2009).

Since methodological considerations may influence the outcome of the CPP test (Buccafusco, 2000; Cheer *et al.*, 2000; Tzschentke, 2007), a validation in this regard was crucial in order to confidently establish the method in our laboratory. Thus, the aim was to validate the CPP paradigm in our laboratory utilising a drug that has known rewarding properties in rodents, in

Addendum A Validation of the conditioned place preference (CPP) paradigm

this case methamphetamine (MA). MA has been extensively studied in the CPP paradigm and found to produce a significant preference for the drug-paired compartment after conditioning with dosages ranging from 1 - 2 m/kg (Pickens *et al.*, 1978; Tuazon *et al.*, 1992; Zakharova *et al.*, 2009; Logan *et al.*, 2016; Toussaint, 2017) and no significant differences in the obtained place preference after sub-acute (day 8) or sub-chronic (day 15) exposure (Bryant *et al.*, 2012) MA was therefore identified as the drug of choice with which to undertake the CPP validation process.

A1.2 Drug exposure protocol

MA was administered as methamphetamine hydrochloride (Sigma-Aldrich, Johannesburg, South Africa), at a dose of 1 mg/kg, i.p. dissolved in saline and administered at 9:00 am on days of drug exposure. This dosage was based on a previous study indicating preference for the drug-paired compartment in the CPP test (Zakharova *et al.*, 2009). Animals were exposed to MA on the mornings of day 1, 3 and 5 with vehicle administered on days 2, 4 and 6 of drug exposure at 9:00 each morning, according to Zakharova and colleagues. Previous studies have found that neither 2% methylcellulose solution nor saline produce a place preference in animals after conditioning (Ashby *et al.*, 2003; Zakharova *et al.*, 2009; Gatch *et al.*, 2013), while 2% methylcellulose solution was the vehicle of choice for efavirenz in the main study. The control group animals therefore received 2% methylcellulose solution as vehicle control on the aforementioned days.

A1.3 Animals

Male, adult Sprague-Dawley (SD) rats (200g - 250g, Vivarium, North West University) were randomized to 12 rats per exposure group with a total of 2 groups. Animals were bred and housed under conditions described in chapter 3 (see section 3.2.1). Ethical approval for this study was obtained (NWU - 00267- 16- S5) from the North-West University animal research ethics committee (AnimCare) (NHREC reg. number AREC-130913-015) before commencing with the study. Of importance to note is that no additional animals than that described in Chapter 3 were used, thus the data of the vehicle and MA exposure groups in the CPP presented in this addendum are the same as that presented in Chapter 3.

A1.4 Methodology:

Condition testing was conducted in a three-compartment apparatus, constructed of plexiglass, containing three compartments separated by guillotine doors. As opposed to the two compartment model, the three compartment design rules out the bias of the animals towards the compartment it was placed in (by the researcher) at the beginning of testing (Bardo & Bevins, 2000; Buccafusco, 2000).

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The validation therefore took place in a three compartment CPP box, designed and manufactured by the instrument manufacturing services of the North West University (NWU) according to previous described protocols (Bardo & Bevins, 2000; Buccafusco, 2000). The two large outer compartments (35 X 35 x 24 cm) were separated by a smaller centre “choice” compartment (15.5 X 19.5 cm), which was used on the habituation and test days (Mueller & Stewart, 2000). The two outer compartments were visually different and had different textures on the floor: one compartment had black and white striped walls and a wire mesh floor with the other compartment presenting with black walls and plexiglass flooring, indicated in Figure A-1. The middle compartment had grey walls and plexiglass flooring (see Figure A-1). The apparatus was constructed according to previously published literature (Meehan & Schechter, 1998; Bardo & Bevins, 2000; Buccafusco, 2000; Duvauchelle *et al.*, 2000; Braida *et al.*, 2004; Tzschentke, 2007; Schlussman *et al.*, 2008; Zakharova *et al.*, 2009).

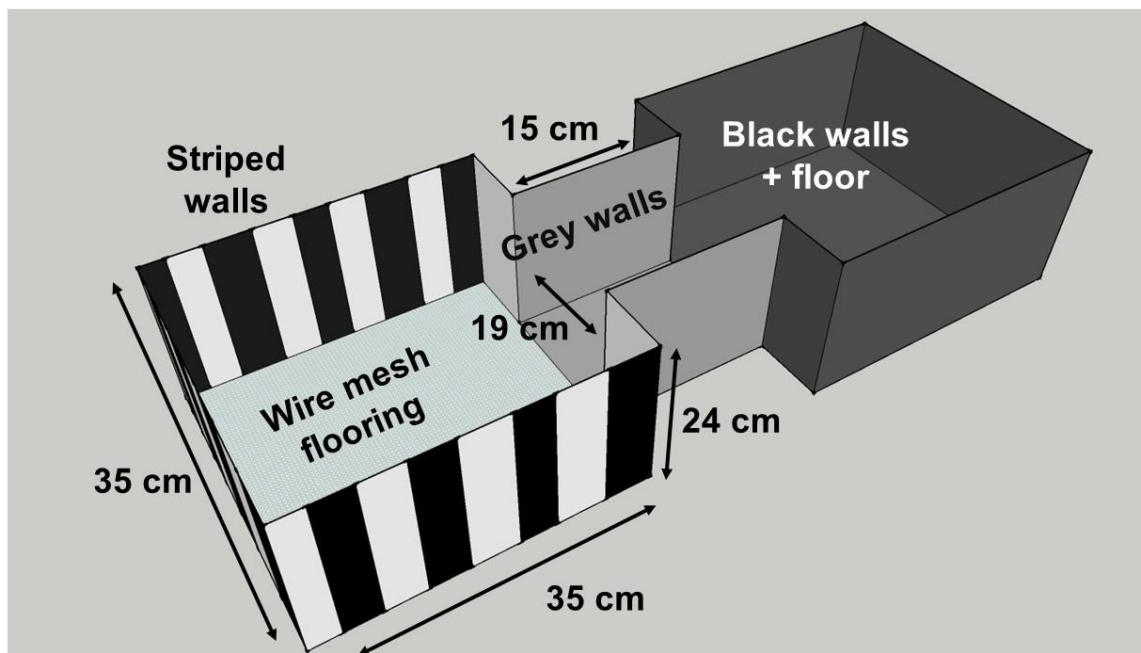


Figure A-1: Illustration of the three-compartment conditioned place preference (CPP) apparatus manufactured at the NWU and used for the CPP validation process in our laboratory.

For the validation process, the CPP test was performed over a period of 8 days in order to simulate the design features of the sub-acute study, explained in Chapter 3. The study was divided into 3 stages, adapted from (Buccafusco, 2000; Braida *et al.*, 2004) (see Table A-1). The first stage was the habituation stage where animals were allowed free access to all 3 compartments for 20 minutes. Here the baseline data so provided was assessed as the time spent in each compartment. The compartment in which the least time was spent was then

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assigned as the “drug-paired compartment” with the most preferred compartment assigned as the “vehicle-paired compartment”. This was done to prevent any unintended bias by the animal for a specific compartment.

The second stage was conditioning, where the animals were then injected with the test-drug and subjected to the drug-paired compartment, as identified previously, for 20 minutes on drug exposure days. On vehicle exposure days, animals were subjected to the vehicle-paired compartment for 20 minutes. Only the outer compartments were used during the conditioning period.

The last stage involved post conditioned testing, wherein the animals were allowed free access to all the compartments on the testing days for 20 minutes, with the time spent in each compartment recorded and quantified. More time spent in the drug-paired compartment (preference) was evident of a rewarding drug and less time spent in the drug-paired compartment (aversion) was evident of an aversive drug (Bardo & Bevins, 2000; Buccafusco, 2000; Braida *et al.*, 2004).

Table A-1: The CPP validation procedure; alternating between MA and vehicle exposure each day while the control group only received vehicle exposure throughout.

Day	Exposure procedure	Time spent in compartment (min)	Test procedure
1	No drug	20	Habituation: Free access to all compartments
2	Drug	20	Conditioning: Least preferred compartment
3	Vehicle	20	Conditioning: Most preferred compartment
4	Drug	20	Conditioning: Least preferred compartment
5	Vehicle	20	Conditioning: Most preferred compartment
6	Drug	20	Conditioning: Least preferred compartment
7	Vehicle	20	Conditioning: Most preferred compartment
8	No drug	20	Testing: Allowed free access to all compartments

All behaviour was recorded under dim white light (30 lux) with a digital video camera and scored using EthoVision© XT software (Noldus Information Technology, Wageningen, Netherlands) to assess the time spent in the respective compartments prior and post

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conditioning. Data is presented as the difference in time spent in the drug-paired compartment utilising the formula: *Time spent in drug paired compartment during post-test (s) – Time spent in drug-paired compartment during habituation (s)* (Malanga *et al.*, 2007).

A1.5 Statistical analysis

Graphpad Prism version 7 for windows (Graphpad software, San Diego, USA) and SAS/STAT® Software was used for the statistical analysis and graphical presentations, with all statistical analysis done under the guidance of the Statistical Consultation Service of the North-West University. To compare the difference in time spent in the drug-paired compartment of the MA and vehicle exposure groups, an unpaired student's t-test was performed. Data are expressed as the mean \pm standard error of the mean (SEM), with a p value of < 0.05 deemed statistically significant.

A1.6 Results:

An unpaired student's t-test revealed that animals exposed to sub-acute MA (1 mg/kg) presented with a significant increase in the time spent in the drug-paired compartment compared to a vehicle control group ($p = 0.0009$)

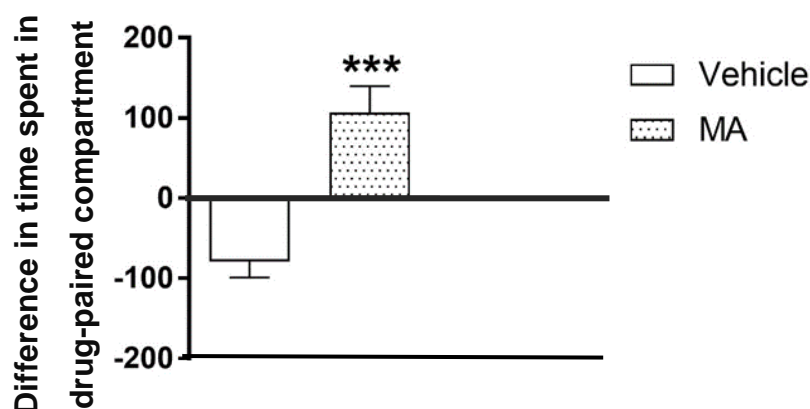


Figure A-2: Conditioned place preference test in animals exposed to sub-acute MA (1mg/kg) and a vehicle control, respectively. Data presented as: time spent in drug paired compartment during post-test (s) – Time spent in drug-paired compartment during habituation. *** $p < 0.001$ vs Vehicle (Unpaired student's t-test)

A1.7 Discussion

The literature suggests that MA (1 mg/kg) has significant rewarding properties in animals using a 6 day dosing strategy, evident in an increase in the time spent in a drug-paired compartment after conditioning in a CPP paradigm (Zakharova *et al.*, 2009). Furthermore, a previous indicated that after conditioning with MA over a period of 15 days, there was a significant increase in time spent in the drug-paired compartment, however, there were no significant differences in preference for the drug-paired compartment between days 8 and 15, indicating no changes in side-preference after sub-acute and sub-chronic exposure. In the present study we aimed to validate the CPP methodology in our laboratory by exposing animals sub-acutely to MA at 1mg/kg and compare it to animals only exposed to a vehicle. Our CPP paradigm indicated that sub-acute MA at 1 mg/kg induced a significant preference for the drug-paired compartment compared to a vehicle control in line with other studies. Thus, the aim to validate the CPP paradigm in our laboratory for a sub-acute and sub-chronic study was reached.

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Zakharova, E., Leoni, G., Kichko, I. & Izenwasser, S. 2009. Differential effects of methamphetamine and cocaine on conditioned place preference and locomotor activity in adult and adolescent male rats. *Behavioural brain research*, 198(1):45-50.

Addendum B

Neurochemical and peripheral analysis

This addendum includes additional information regarding the methods and procedures followed in determining the concentrations of regional brain (frontal cortex, striatum, and hippocampus) monoamines and lipid peroxidation and the percentage superoxide dismutase (SOD) in the plasma of animals exposed sub-chronically to either efavirenz at 5mg/kg or a vehicle control.

The methods described in this addendum are maintained and validated by the Analytical Technology Laboratory (ATL) of the Centre of Excellence for Pharmaceutical Sciences (PharmaGen) of the North-West University, Potchefstroom Campus. The high performance liquid chromatography (HPLC) methods were previously validated (Harvey *et al.*, 2006), therefore only system suitability was done for this study. The enzyme-linked immune sorbent assay (ELISA) kits were done according to the manufacturer's instructions and thus a summary of the methods and materials will be discussed.

The addendum consists out of:

- Quantification of monoamines and their metabolite levels in brain samples
- Quantification of %SOD in the plasma
- Quantification of regional brain lipid peroxidation

B1.1 HPLC method for assessing regional brain monoamines

B1.1.1 Chromatographic conditions

The following specific parameters (including instrument, column, flowrate, injection volume and wavelength) were set and used for the assessment of the regional monoamines (see Table B-1).

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	Luna C18 (2) column, 150 x 4.6 mm, 2.6µm, 100 Å pores, (Phenomenex, Torrance, CA).
Flow rate	1.0ml/ min
Injection volume	20 µl
Diode Array Detector (DAD) settings	Wavelength set at 245 nm
Run Time	± 25 minutes

B1.1.2 Mobile phase preparation

0.1 M sodium formate buffer, 0.5 mM ethylene-diaminetetraaceticacid (EDTA dinatriumsalt Na₂EDTA), 5 mM sodium heptane sulphonic acid, 5% v/v acetonitrile. The pH of the mobile phase was set at ± pH 4.10 using orthophosphoric acid (85%).

B1.1.3 Standard preparation

The standard solution contains precisely known concentrations of the monoamines which was used to determine the unknown concentrations in the brain samples.

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

- Preparation of *solution A*:

0.5 mM Sodium metabisulphite

0.3 mM Na₂EDTA

0.1 M Perchloric acid (60% strong solution).

1. Dissolved 0.09505 g sodium metabisulphite and 0.111672 g Na₂EDTA in 800 ml distilled water.
2. Add 10.87 ml perchloric acid to the above solution and made it up to 1000 ml with distilled water.

Addendum B: Neurochemical and peripheral analysis

- Preparation of stock solutions:

Dissolved 1mg of each analyte in 10ml of *solution A*, this yields a concentration of 100µg/ml for each analyte. Stock solutions of each monoamine: noradrenaline (NA), 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindole-3-acetic acid (5-HIAA) and serotonin (5-HT) were prepared for the preparation of the standard solutions as indicated in Table B-2.

Table B-2: Preparation of stock solutions

Monoamine/metabolite standard	Raw materials	Methodology
Internal standard	Isoprenaline	Dissolve 1 mg in 10 ml <i>solution A</i> . This serves as the internal standard (IS) stock solution. 30 µl of this stock solution is made up to 2 ml with <i>Solution A</i> , which serves as the working internal standard producing a concentration of 1500 ng/ml.
Dopamine (DA)	3-Hydroxythyramine hydrochloride = 189.64 MW	Dissolve 1.24 mg in <i>solution A</i> (100 µg/ml)
Serotonin (5-HT)	5-Hydroxytryptamine creatinine sulphate = 405.43 MW	Dissolve 2.30 mg in <i>solution A</i> (100 µg/ml)
3,4-Dihydroxyphenylacetic acid (DOPAC)	3,4-Dihydroxyphenylacetic acid = 168.15 MW	Dissolve 1 mg of DOPAC in <i>solution A</i> (100 µg/ml)
5-Hydroxyindole-3-acetic acid (5-HIAA):	5-Hydroxyindole-3-acetic acid = 191.19 MW	Dissolve 1 mg 5-HIAA in <i>solution A</i> (100 µg/ml)
Noradrenaline (NA)	L-Noradrenaline hydrochloride = 205.6407 MW	Dissolve 1.22 mg in <i>solution A</i> (100 µg/ml)

From the stock solutions a concentration range of 10 - 200 ng/ ml standards were prepared in distilled water (see Table B-3) and stored between 2 – 8 °C.

Table B-3: Preparation of standard solutions

Standard number	Concentration (ng/ml)	Dilution Volume	+	Solution A	=	Total Volume
1	10	200µl	+	1800 µl	=	2 ml
2	25	10µl	+	1990 µl	=	2 ml
3	50	20µl	+	1980 µl	=	2 ml
4	100	40µl	+	1960 µl	=	2 ml
5	150	60µl	+	1940 µl	=	2 ml
6	200	80µl	+	1920 µl	=	2 ml

B1.1.4 Sample preparation

Following dissection of rat brain, tissue was snap frozen in liquid nitrogen and stored at -80°C until the day of analysis.

- On the day of analysis, samples were weighed, thawed and 1 ml of solution A was added to each Eppendorf tube.
- The tissue in each tube was then sonicated (2 x 12 seconds, amplitude of 14 µ)
- The tubes were allowed to stand on ice for 20 minutes to complete perchlorate precipitation of protein and extraction of monoamines.
- Samples were centrifuged at 4°C for 20 minutes at 16 000 rpm (24 000 g).
- The supernatant was removed and 1 drop/ml of 10 M potassium acetate was added, the pH of the sample was adjusted to 5.
- 200 µl of the supernatant was pipetted into another Eppendorf tube.
- 20 µl of the internal standard, isoprenaline, was added to the sample.
- The final sample was vortexed and centrifuged, with 20 µl injected onto the HPLC column.
- The results were expressed as ng/g wet weight of tissue.

B1.1.5 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 in the sample.

The linearity used in this validation process comprised of the following standard concentrations: 10, 25, 50, 100, 150 and 200 ng/ ml. The linear regression value determined for each analyte is presented in figures B-1 – B-3. The levels of the noradrenaline metabolite, MHPG (3-hydroxy methoxy phenylglycol), was below the limit of detection for this system.

Addendum B: Neurochemical and peripheral analysis

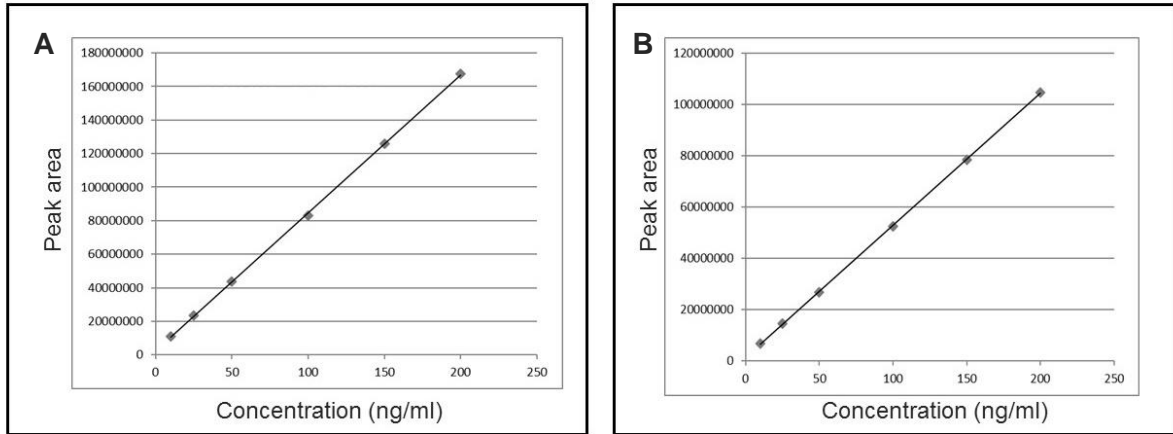


Figure B-1: Standard linear regression graph of **(A)** dopamine (DA). $R^2=0.999$ and **(B)** dihydroxyphenylacetic acid (DOPAC) ($R^2=0.999$)

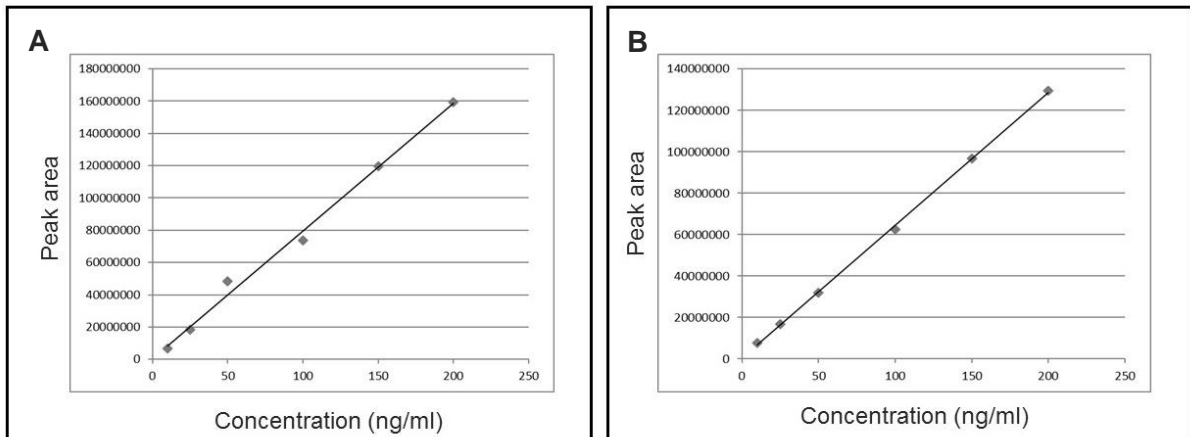


Figure B-2: Standard linear regression graph for **(A)** serotonin (5-HT) ($R^2=0.993$) and **(B)** 5-Hydroxyindole-3-acetic acid (5HIAA) ($R^2=0.999$)

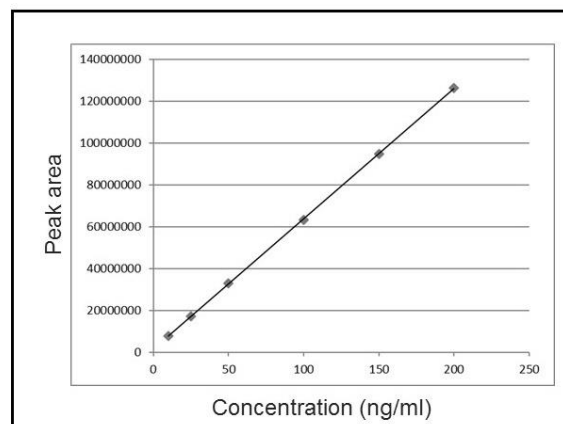


Figure B-3: Standard linear regression for noradrenaline (NA). ($R^2=0.999$)

The acceptable criteria for regression (R^2), the coefficient of determination for biomolecules, must be at least 0.95 or greater.

B1.1.6 Selectivity and specificity

The selectivity and specificity refers to a specific method as being capable of accurately analysing specific components in the presence of other components with reference to metabolites and other biological substances. The chromatograms of the blank sample, monoamines and related metabolites in the specific brain regions are indicated in Figures B-4 – B-7

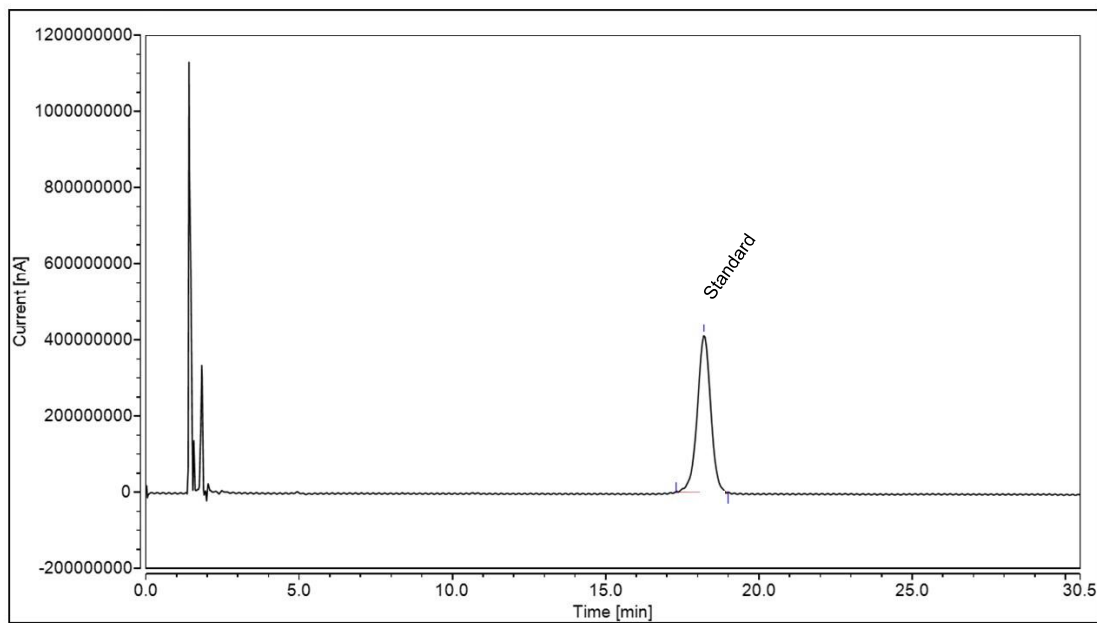


Figure B-4: Chromatogram of blank sample, measured in mAU with a retention time of ± 17 minutes.

Addendum B: Neurochemical and peripheral analysis

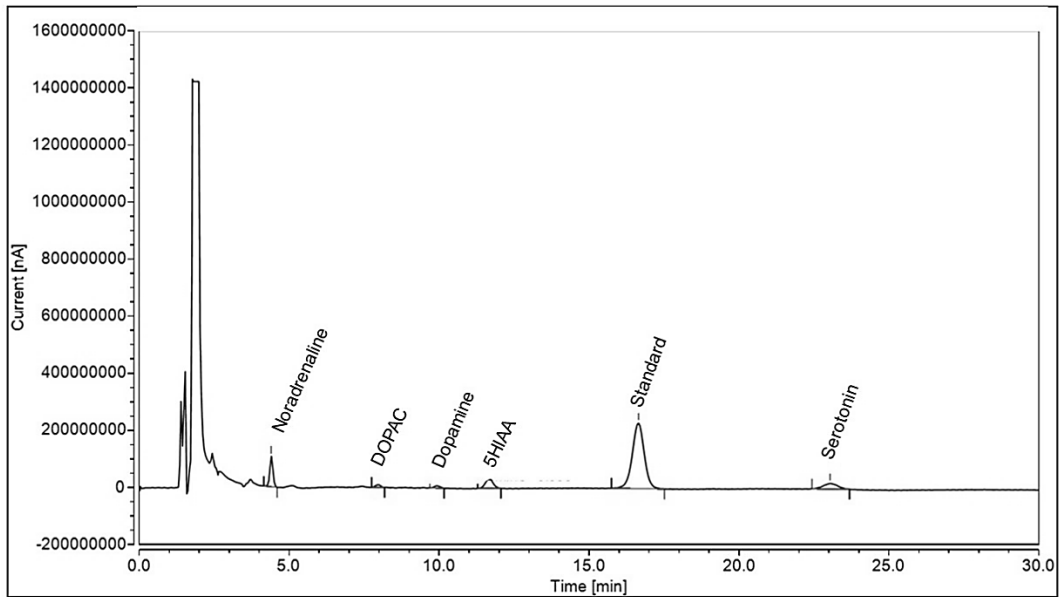


Figure B-5: Chromatogram of frontal cortical monoamines measured in mAU with a retention time of ± 17 minutes. DOPAC: 3,4-dihydroxyphenylacetic acid. 5HIAA: 5-hydroxyindoleacetic acid

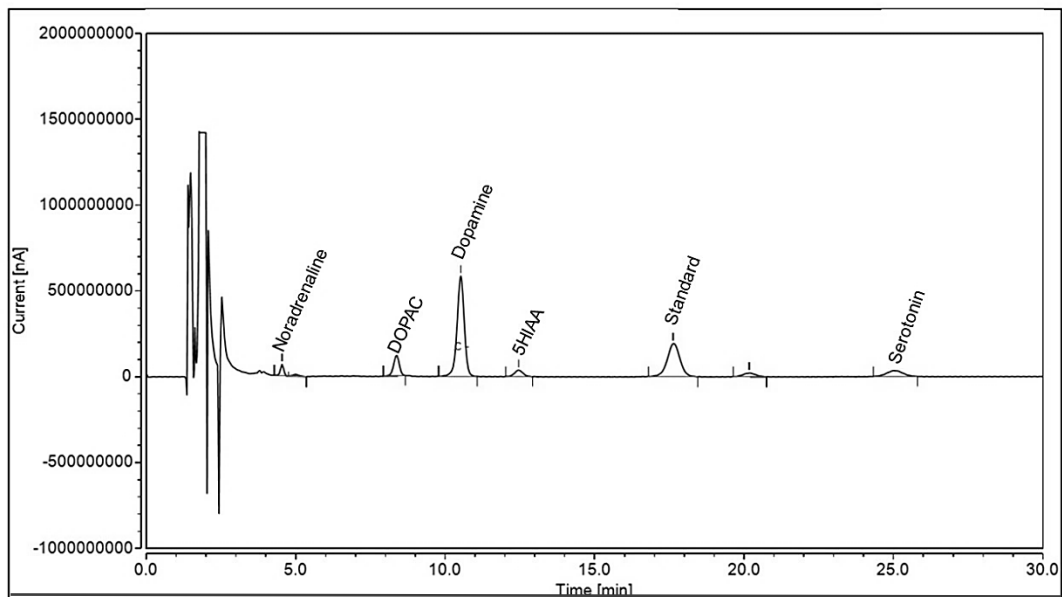


Figure B-6: Chromatogram of striatal monoamines measured in mAU with a retention time of ± 17 minutes. DOPAC: 3,4-dihydroxyphenylacetic acid. 5HIAA: 5-hydroxyindoleacetic acid

Addendum B: Neurochemical and peripheral analysis

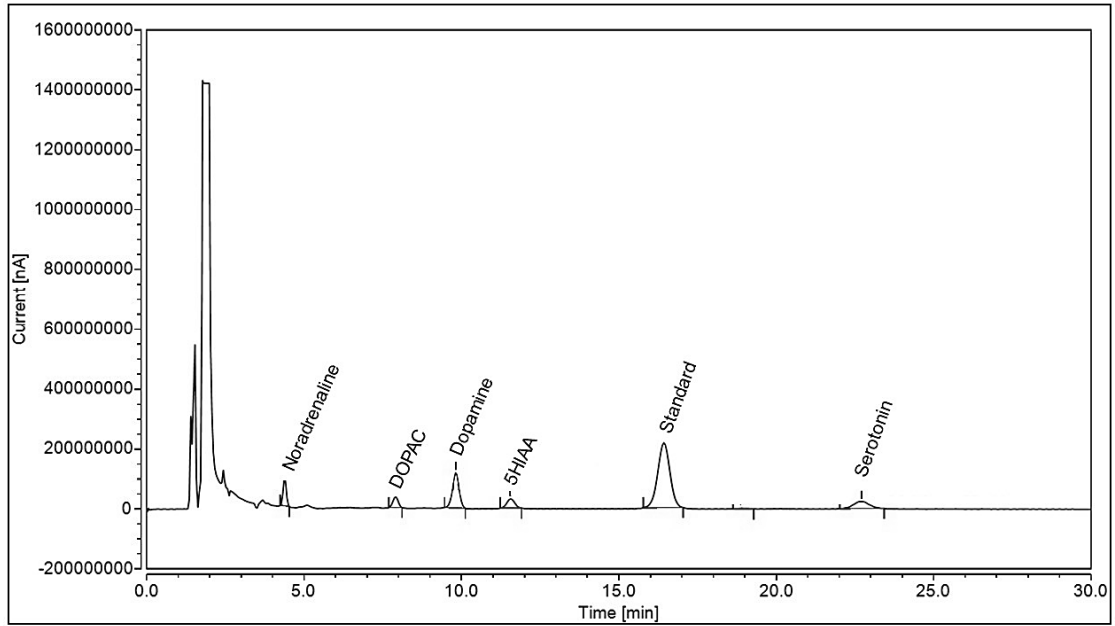


Figure B-7: Chromatogram of hippocampal monoamines measured in mAU with a retention time of ± 17 minutes. DOPAC: 3,4-dihydroxyphenylacetic acid. 5HIAA: 5-hydroxyindoleacetic acid

This method proved effective, based on its adequate selectivity and sensitivity as evidenced by the lack of overlapping peaks between the different compounds that were analysed in the above chromatographs.

B1.2 Quantification of %SOD in plasma

B1.2.1 Introduction

Superoxide dismutase (SOD) is an anti-oxidative enzyme responsible for catalysing the conversion of superoxide anion to hydrogen peroxide and one oxygen molecule. The assay kit utilizes water soluble tetrazolium salts (WST) - 1 which forms a dye upon reduction with O_2^- . The reduction rate of O_2^- is directly related to xanthine oxidase activity (XO) (See Figure B-8 for details) (Pereira *et al.*, 2009).

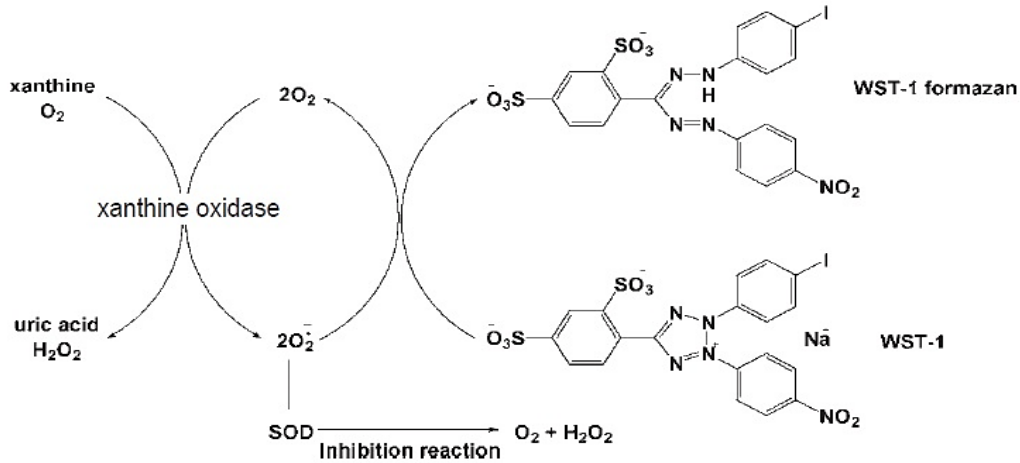


Figure B-8: Superoxide (O_2^-) reduction process. Xanthine oxidase generates the superoxide anion which is reduced by superoxide dismutase (SOD). This process leads to the reduction of water soluble tetrazolium (WST)-1 to WST formazan which absorbs light at 450 nm. (Pani *et al.*, 2004).

Table B-4: Content of the %SOD ELISA KIT (BioVision™ Superoxide Dismutase activity assay kit, USA, catalogue number: K335-100)

Component	
WST solution	1 ml
SOD enzyme solution	20 ml
SOD assay buffer	20 ml
SOD dilution buffer	10 ml

B1.2.2 Sample preparation

Trunk blood was collected into pre-chilled heparin tubes, centrifuged at 20 000 x g at 4 °C for 10 min and the plasma stored at -80°C until the day of analysis

B1.2.3 %SOD ELISA kit procedure

*Refer to Table B-5 for the amount of the specific solution, as supplied by the manufacturer, to be placed in each well.

1. 20 µl of the sample solution was added to each well for sample and for blank 2. 20 µl of ddH₂O (double distilled water) was then added to the wells for blank 1 and blank 3.
2. 200µl of the water soluble tetrazolium (WST) Working Solution was added to each well.
3. 20µl of the Dilution Buffer was added to each Blank 2 and Blank 3 well.
4. 20µl of the Enzyme Working solution was added to each sample and Blank 1 well and thoroughly mixed.

Note: since the superoxide is released immediately after the addition of the Enzyme working Solution to each well, a multiple channel pipette was used to avoid reaction time lag of each well.

5. Plates were incubated at 37°C for 20 minutes.
6. The absorbance was read at 450 nm using a microplate reader.
7. The % SOD activity (inhibition rate %) was calculated according to following the equation:

$$\text{SOD Activity (inhibition rate \%)} = \frac{(\text{Ablank1} - \text{Ablank3}) - (\text{Asample} - \text{Ablank2})}{(\text{Ablank1} - \text{Ablank3})}$$

Table B-5: The specific amount of each solution added to a well on a 96 well plate, for sample, blank 1, 2 and 3 respectively.

	Sample	Blank 1	Blank2	Blank 3
Sample solution	20 µl		20 µl	
ddH ₂ O		20 µl		20 µl
WST Working solution	200 µl	200 µl	200 µl	200 µl
Enzyme Working solution	20 µl	20 µl		
Dilution Buffer			20 µl	20 µl

B1.2.4 Calibration

In order to monitor the SOD activity in plasma, a calibration curve was set up using standard solutions of 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml.

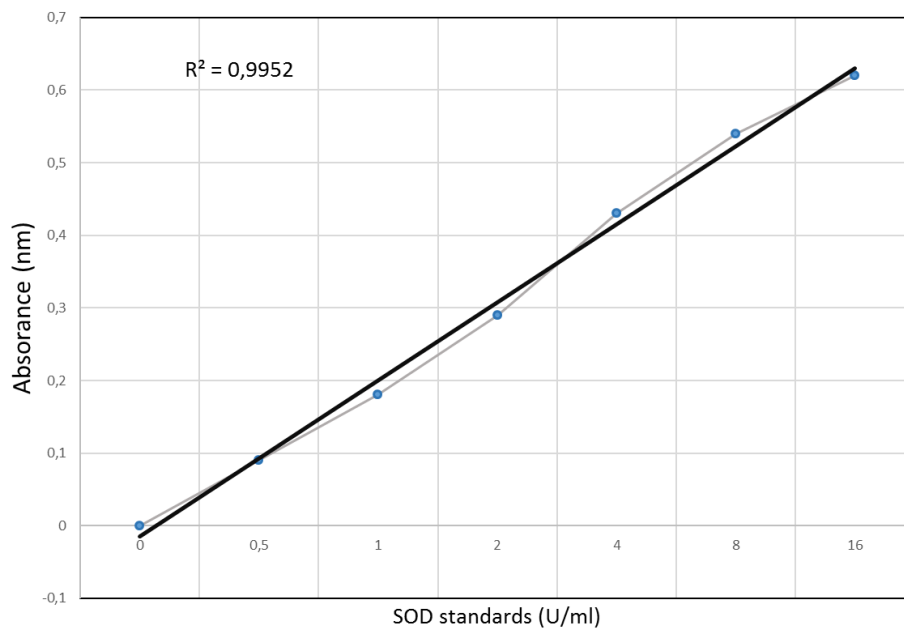


Figure B-9: Calibration curve of the SOD standard solutions, with R² of 0.9952.

B1.3 Quantification of regional lipid peroxidation

The quantification of frontal cortical and striatal lipid peroxidation was followed according to the package insert of Thiobarbituric Acid Reactive Substances (TBARS) assay kit, as directed by the manufacturer

www.rndsystems.com/products/tbars-parameter-assay-kit_kge013

B1.3.1 Introduction

Oxidizing agents can alter lipid structure, creating lipid peroxides that result in the formation of malondialdehyde (MDA), which can be measured as TBARS (Benzie, 1996). This kit is based on the principle of MDA reacting with thiobarbituric acid (TBA) in the presence of heat and an acid to produce a coloured product that is able to absorb light at 532 nm, corresponding to the levels of lipid peroxidation in the sample (see Figure B-10).

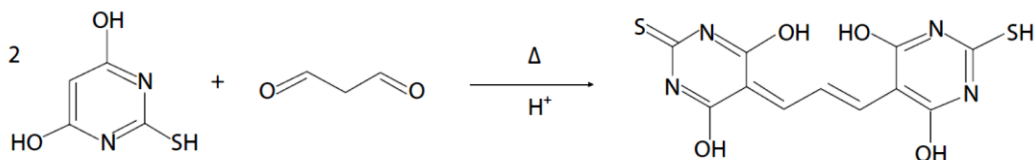


Figure B-10: Thiobarbituric acid (TBA) in the presence of heat (Δ) and an acid (H^+) produces a coloured product able to absorb light (Benzie, 1996)

B1.3.2 Materials

- Uncoated plate
- TBA reagent
- TBARS standard
- TBARS acid reagent
- Plate sealers
- Microplate reader capable of measuring absorbance at 530-532 nm.
- 45-50 °C incubator.
- Microcentrifuge capable of $\geq 12,000 \times g$.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Micro-centrifuge tubes for acid treatment.
- Test tubes for dilution of standards
- Sonicator

Addendum B: Neurochemical and peripheral analysis

- Phosphate buffered solution (BPS)

B1.3.3 Sample preparation

PBS was prepared by dissolving 200g potassium chloride (KCl) and 22.5g disodium phosphate (Na_2HPO_4) in 2.5l distilled water. One part of the PBS solution was diluted with 9 parts distilled water to obtain a 0.01mM PBS solution.

1. Samples were left to thaw on ice and weighed
2. A 10% w/v solution was made with the brain samples in the prepared PBS at a pH of 7.4 and homogenised by ultrasonification. (12 second bursts in duplicate at a 14 μ amplitude)
3. Samples were then centrifuged at 800 x g for 10 minutes at 4° C and the supernatant used for the acid treatment.
4. 300 μ L of the sample (supernatant) and 300 μ L TBARS Acid was added to a micro-tube and incubated for 15 min at room temperature
5. Samples were again centrifuged at $\geq 12,000$ x g for 4 min and supernatant removed.

The concentration read off the standard curve must be multiplied by the dilution factor of 2

B1.3.4 Standard preparation:

1. Standards were converted to MDA by adding 100 μ l of TBARS Standard to 200 μ l of TBARS Acid Reagent.
2. All standards were left to rest for at least 30 minutes with agitation. This produced a stock solution of 167 μ M.
3. 900 μ l of deionized water was pipetted into the 16.7 μ M tube.
4. 500 μ l of deionized water was pipetted into the remaining tubes.
5. Stock solution was used to produce a dilution series (see Figure B-11).
6. The 16.7 μ M standard served as the high standard and deionized water served as the 0 μ M standard.

Addendum B: Neurochemical and peripheral analysis

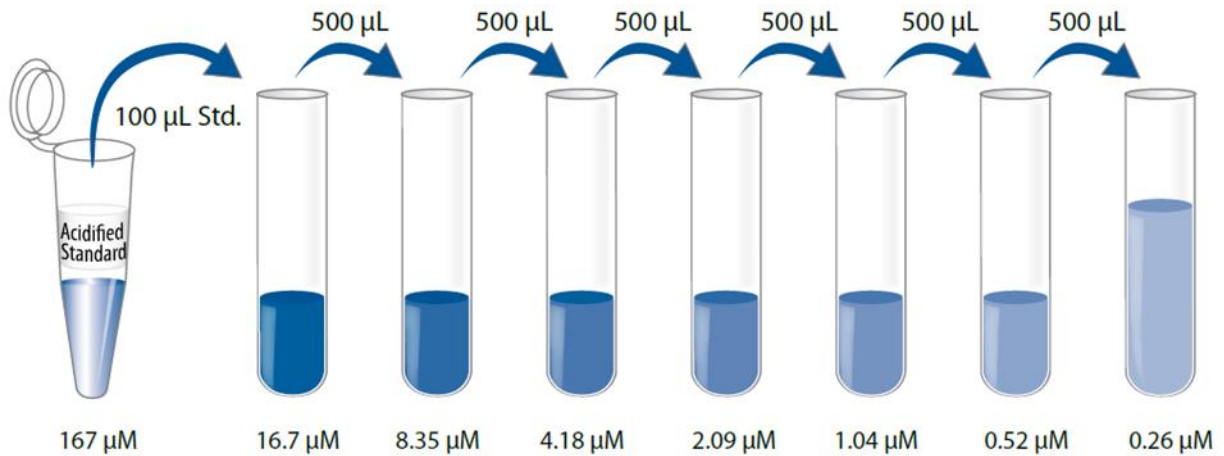


Figure B-11: TBARS standard series of dilutions in µM (Adapted from TBARS assay kit (Paramater, 2017))

B1.3.5 Assay procedure:

1. 150 µl of standards and samples was added to each well.
2. 75 µl of TBA reagent was added to each well.
3. Optical density of each well was pre-read using a microplate reader set to 532 nm.
4. The microplate was incubate for 2-3 hours at 45-50 °C.
5. The optical density of each well was read using a microplate reader set to 532 nm.
6. Pre-reading was subtracted from the final reading to correct for the sample's contribution to the final absorption at 532 nm.

B1.3.6 Results

Microsoft Excel was used generate a linear curve. Seeing that samples have been diluted, the concentration read from the standard curve was multiplied by the dilution factor, 2.

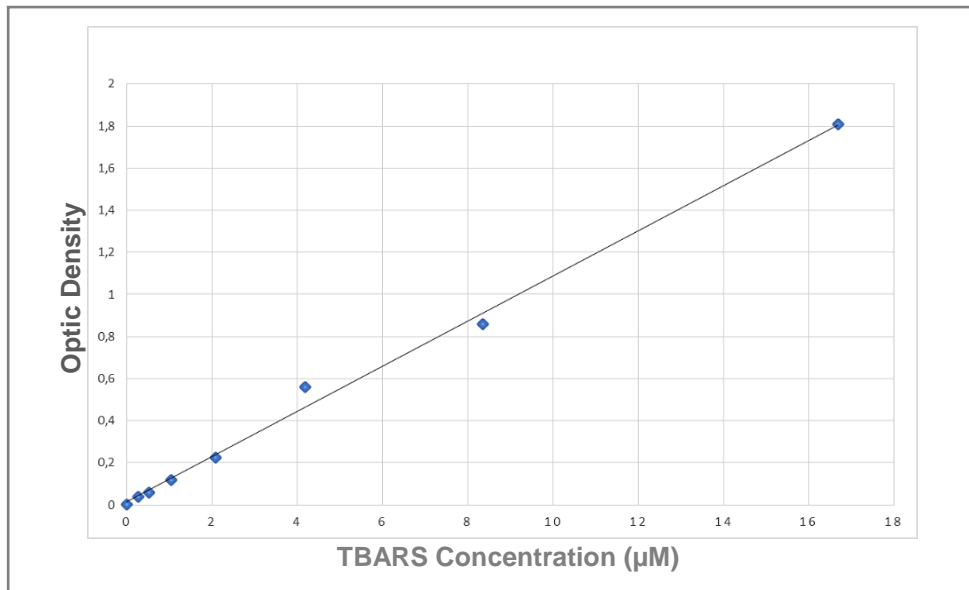


Figure B-12: Standard linear curve for TBARS using concentrations: 0 µM, 0.26 µM, 0.52 µM, 1.04 µM, 2.09 µM, 4.18 µM, 8.35 µM, 16.7 µM. Optic density was measured at 532 nm and data calculated using a standard curve with regression value ($y=0,1073x + 0,0145$ $R^2 = 0,9954$)

B1.4 Conclusion

The discussed procedures for quantification of regional brain monoamine, lipid peroxidation and peripheral SOD were applied in the current study as described in detail in chapter 3.

B1.5 References

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Addendum C

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Addendum D

Authors' approval letters



29 November 2017

Dear examiner

**MSc DISSERTATION – J FOURIE
PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES**

As study leader and senior corresponding author on the article presented in Chapter 3, first authored by Mr. Jaco Fourie, I hereby approve that the concept manuscript listed below be included as part of the requirements for fulfilment of the MSc. degree, and that this manuscript may be submitted for examination purposes by the candidate.

The article is as follows:

Chapter 3

Evaluation of sub-acute and sub-chronic efavirenz exposure on neurochemical and oxidative stress markers and addictive-like behaviours in rats

Sincerely,



Marisa Möller, PhD

29 November 2017

Dear examiner

**MSc DISSERTATION – J FOURIE
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As co-author on the article presented in Chapter 3, first authored by Mr. Jaco Fourie, I hereby approve that the concept manuscript listed below be included as part of the requirements for fulfilment of the MSc. degree, and that this manuscript may be submitted for examination purposes by the candidate.

The article is as follows:

Chapter 3

Evaluation of sub-acute and sub-chronic efavirenz exposure on neurochemical and oxidative stress markers and addictive-like behaviours in rats

Sincerely,



Brian H. Harvey, PhD
Co-study leader