The long-term effects of methamphetamine on depressive-like behaviour and neuroplasticity in stress-sensitive rats

M Mouton

24295906

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Study Leader: Prof CB Brink

Co-Study Leader: Prof BH Harvey

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Abstract

Methamphetamine (METH) abuse has become a fast growing drug problem that has developed into a global epidemic. In fact, METH is one of the most commonly abused substances with an estimated 35 million abusers worldwide and is said to be the second most popular illicit drug. The Western Province of South Africa has seen a dramatic increase in drug abuse in recent years where METH is the primary or secondary drug of abuse. Interestingly, more than 50% of these individuals are under the age of 20 years. The longer duration of euphoric effects of METH has attracted many users away from cocaine in favour of METH.

In addition to the rapid euphoric effect of METH, the direct short-term effects include arousal, reduced fatigue, an increase in blood pressure, reduced appetite as well as sustained attention. Chronic METH abuse may result in debilitating and long-lasting effects that includes mood disorders such as depression. Studies suggest a strong relationship between exposure to adverse environmental factors early in life and the later development of a neuropsychiatric disorder, such as depression. However, these severe consequences do not seem to invoke cessation of the drug. The euphoric and addictive properties of METH causes users to abuse the drug with an increase in frequency and dose, even though it might not have been their original intention.

The primary objective of this study was to investigate the effect of early-life administration of METH to stress-sensitive (Flinders Sensitive Line - FSL) and control (Flinders Resistant Line - FRL) rats on depressive-like behaviour and regional brain monoamine levels later in life.

The study implemented a sixteen-day period for administration of METH or a vehicle control from postnatal day 19 (PnD19) to postnatal day 34 (PnD34). The latter developmental stage corresponds to pre-adolescence in the rat when neurological development are similar to that seen in human adolescents, and represents the stage when drug abuse is most common in humans. Chronic dosing of METH and saline was performed twice daily at 09:00 and at 15:00. The animals received a sub-cutaneous (SC) escalating dose regimen of METH during the 16 day period (mimicking binging behaviour in humans), with every dose escalating in increments of 0.2 mg/kg from 0.2 mg/kg to 6.0 mg/kg. The study then investigated whether early-life administration of METH would cause depressive-like behaviours directly after the injection period (immediate drug effects before withdrawal on PnD35) or later in life (after the withdrawal period in early adulthood on PnD60). The behavioural effects were assessed in a

battery of tests and thereafter the rats were sacrificed and the frontal cortex removed and snap frozen for later analyses of altered neurochemistry.

The study demonstrated that chronic METH treatment during pre-adolescence induces significant behavioural changes related to depression in humans directly after the injection period (PnD35) and later in life (PnD60). The animals displayed antidepressant-like behaviour in the forced swim test (FST) before withdrawal, yet a depressogenic effect was observed 25 days post-withdrawal. This effect also seems to be additive to the congenital depressive-like phenotype of FSL rats, suggesting a role for genetic susceptibility. This observation would be in line with the two-hit hypothesis of depression, suggesting that the manifestation of depression will result when a genetic predisposition is followed by an environmental stressor (i.e. METH) later in life. The data suggests a working hypothesis that individuals that already have a predisposition to depression may be more susceptible to developing depression when abusing METH. The fact that the FSL control rats were more immobile than FRL control rats also confirmed the face validity of the FSL genetic rat model of depression.

Locomotor activity assessment indicated that METH treatment decreased locomotor activity in FSL and FRL rats compared to their vehicle controls on PnD35 but not on PnD60. It is important to note that the effects observed in locomotor activity could not have contributed to the immobility observed in the FST, confirming that the immobility in the FST indeed reflects psychomotor and not locomotor effects. The study also demonstrated that METH significantly lowers social interaction behaviour in both FRL and FSL rats, both immediately following drug treatment (PnD35) and after withdrawal (PnD60). It is therefore clear that this effect of METH is long-lasting, putatively related to neurodevelopmental effects. In addition, the rats investigated the familiar object for a greater amount of time in the novel object recognition test (nORT) on PnD35 and PnD60 and may be the result of loss of recognition memory for the familiar object. This data confirms that METH results in cognitive memory deficits probably due to sustained adverse neurodevelopmental effects.

Neurochemical analyses of the frontal cortex indicated decreased serotonin (5-HT) and norepinephrine (NE) levels on PnD35. METH is widely recognised for its pro-inflammatory effects, while the reduced 5-HT levels observed may have been the result of an increase in circulating pro-inflammatory cytokines. Neurochemical analyses provided thought-provoking data concerning the role of the permissive hypotheses of depression, indicating that dopamine (DA) is most likely not responsible for the behavioural effects observed, at least under the current study conditions, whereas 5-HT is decidedly more involved than expected. The data also suggest that depletion in NE plays a role in the development of depressive-like

behaviours following METH exposure. Based on these findings, we propose that disturbances in 5-HT and NE are a crucial mechanism in how METH abuse may precipitate or worsen depressive-like symptoms in individuals who abuse METH. It should be noted that this study does not discard the role of DA in the development of depression after METH exposure, although under the current study conditions it appears that DA does not play a central role.

The current study demonstrated that pre-adolescent exposure to METH can reproduce most of the behavioural changes seen in depressed individuals, and that these behavioural data can be used to identify causal neurochemical factors. Environmental stressors such as METH abuse should be regarded as an additional diagnostic criterion and is relevant to an accumulative risk factor hypothesis. Furthermore, although further study is required, the data suggests that early-life exposure to METH may predispose an individual to mood disorders and behavioural abnormalities later in life.

Keywords: methamphetamine, depression, long-term effects, Flinders Sensitive Line rats, Flinders Resistant Line rats, monoamines, depressive-like behaviour.

Opsomming

Metamfetamien (METH) misbruik het ‘n snel-ontwikkelende dwelm probleem geword met afmetings van ‘n globale epidemie. METH is een van die mees algemeen misbruikte middels en ongeveer 35 miljoen mense misbruik die middel wêreldwyd. METH staan bekend as die tweede mees populêre dwelm in die wereld. In die Wes-Kaap provinsie in Suid Afrika het dwelmmisbruik die afgelope paar jaar geweldig toegeneem met METH as die primêre of sekondêre dwelm van keuse. Van die misbruikers is meer as 50% onder die ouderdom van 20 jaar. Die lang-durende uitwerking van METH veroorsaak dat baie mense METH bo kokaïen verkies.

Addisioneel tot die vinnige euforie wat METH veroorsaak, word ander korttermyn effekte soos opwekking, verminderde moegheid, ‘n verhoging in bloeddruk, ‘n verlaging in eetlus en ook beter konsentrasievermoë waargeneem. Chroniese METH gebruik mag verlammende en lang-termyn effekte veroorsaak wat gemoedsversteuring soos depressie insluit. Vorige studies dui op ‘n sterk verwantskap tussen die blootstelling aan nadelige omgewingsfaktore vroeg in die lewe en die ontwikkeling van ‘n neuropsigiatriese siekte, soos depressie, later in die lewe. Hierdie ernstige nagevolge veroorsaak egter nie die staking van die gebruik van die middel nie. Die euforie en verslawende eienskappe van METH noop die gebruiker om meer van die middel te gebruik, al was dit nie hul oorspronklike bedoeling nie.

Die primêre doelwit van die studie was om die effekte van vroeë-lewe blootstelling aan METH op depressiewe gedrag en brein monoamienvlakke later in die lewe van stres-sensitiewe (Flinders Sensitive Line - FSL) en kontrole (Flinders Resistant Line – FRL) rotte te ondersoek.

Die studie het n sestien-dag behandelings tydperk met METH of soutoplossing gevolg vanaf postnatale-dag 19 (PnD19) tot postnatale-dag 34 (PnD34). Hierdie ontwikkelingstydperk stem ooreen met pre-adolessensie in die rot wanneer neurologiese ontwikkeling soortgelyk is aan adolessensie in die mens en dit is ook die ouderdom wanneer dwelmmisbruik mees algemeen is in mense. Chroniese dosering met METH of soutoplossing is twee keer per dag uitgevoer om 09:00 en om 15:00. Die rotte het ‘n sub-kutaneuse stygende dosering van METH ontvang gedurende die 16 dae van behandeling (wat dosering in mense naboots), elke dosis het gestyg met 0.2mg/kg van 0.2mg/kg tot 6.0mg/kg. Na hierdie behandeling is dit ondersoek of vroeë-lewe blootstelling aan METH depressiewe gedrag sal veroorsaak direk na die behandelingstydperk (onmiddellike dwelm effekte voor ontrekking op PnD35) of later in die lewe (na ontrekking in vroeë volwassenheid op PnD60). Die gedrag van die rotte is

bepaal deur middel van verskeie gedragstoetse, waarna die rotte onthoof en die frontale korteks verwyder en gevries is vir neurochemiese analise.

Hierdie studie het aangetoon dat chroniese METH behandeling gedurende die pre-adolessensie tydperk beduidende gedrags veranderinge veroorsaak wat verband hou met depressie in mense onmiddelik na behandeling (PnD35) asook later in die lewe (PnD60). Voor ontrekking het die rotte gedrag vertoon soorteglyk aan die gedrag wat met die neem van antidepressant waargeneem sou kon word in die geforseerde swemtoets. In teenstelling hiermee, is depressiewe gedrag waargeneem 25 dae na ontrekking. Hierdie effek dra addisioneel by tot die aangebore depressie-agtige fenotipe van die FSL rot, wat ‘n rol van genetiese vatbaarheid voorstel en is dus in lyn met die “two-hit”- hipotese van depressie, wat voorstel dat depressie sal ontwikkel wanneer ‘n individu reeds geneties vatbaar is vir depressie nadat hulle blootgestel word aan nadelige omgewingsfaktore, soos METH, later in die lewe. Ons data is dan in ooreenstemming met hierdie hipotese en impliseer dus dat iemand wat reeds vatbaar is vir depressie meer geneig is om depressie te ontwikkel na dwelmmisbruik. Die feit dat die kontrole FSL rotte meer immobiel was as die kontrole FRL rotte bevestig dat die FSL rot ‘n ware genetiese model van depressie is.

Resultate wat met die lokomotoraktiwiteitstoets verkry is, het aangedui dat METH lokomotoraktiwiteit in die FSL en FRL rotte verlaag het in vergelyking met hul soutoplossing kontrole op PnD35 maar nie op PnD60. Dit is belangrik om te noem dat die effekte wat gesien is in die lokomotoraktiwiteitstoets nie bygedra het tot die immobiliteit wat gesien is in die geforseerde swemtoets nie, wat bevestig dat die waargenome immobiliteit wel die psigomotoriese en nie die lokomotoriese effekte van METH aandui. Die huidige studie het ook bewys dat METH sosiale interaksie verlaag in die FSL en FRL rotte, direk na behandeling op PnD35 en na ontrekking op PnD60. Dus is dit duidelik dat METH lang-termyn effekte veroorsaak wat verband kan hou met neuro-ontwikkelingseffekte. Die rotte het ook die bekende voorwerp vir ‘n langer tydperk verken in die “nuwe voorwerp herkenningtoets” op PnD35 en PnD60 wat moontlik die resultaat mag wees van die verlies van kognisie. Hierdie data bevestig dat METH kognitiewe geheue verlies as gevolg van nadelige neuro-ontwikkelings effekte veroorsaak.

Neurochemiese analise van die frontale korteks het ‘n verlaging in serotonien- en norepinefrienvlakke op PnD35 aangedui. METH is bekend daarvoor dat dit pro-inflammatoriese effekte veroorsaak en die verlaging in serotonien mag die resultaat wees van ‘n verhoging in pro-inflammatoriese sitokiene. Neurochemiese analise het voorts bygedra tot nuwe data wat verband hou met die permissiewe hipotese (“permissive hypothesis”) van depressie. Die neurochemiese resultate het aangedui dat dopamien

waarskynlik nie verantwoordelik is vir die gedrags-effeke nie, of tenminste nie onder die huidige studie omstandighede nie, maar dat serotonien meer betrokke is as wat ons verwag het. Die data dui ook aan dat norepinefrien ‘n belangrike rol in die ontwikkeling van depressiewe gedrag na METH behandeling speel. Ons stel dus voor dat die versteuring in serotonien en norepinefrien die belangrikste meganismes is waarvolgens METH depressie mag veroorsaak of vererger in mense wat METH misbruik. Dit is belangrik om daarop te let dat hierdie studie nie die rol van dopamien in die ontwkkeling van depressie na METH ignoreer nie, maar onder die huidige studie omstandighede blyk dit of dopamien nie ‘n sentrale rol speel nie.

Dus het die huidige studie bewys dat pre- adolessente blootstelling aan METH meeste van die gedrags veranderinge wat gesien word in depressiewe mense kan veroorsaak en dat die data van die gedragstoetse gebruik kan word om die oorsaaklike neurochemiese faktore te identifiseer. Omgewingsstressors, soos METH, moet ook beskou word as addisionele diagnostiese kriteria en dit is relevant tot ‘n kumulatiewe risiko- faktor hipotese. Ten spyte daarvan dat verdere studies noodsaaklik is, bewys die data dat vroeë lewe blootstelling aan METH die vatbaarheid vir gemoedsversteurings verhoog en gedrags afwykings later in die lewe kan veroorsaak.

Sleutelwoorde: metamfetamien, depressie, langtermyn-effekte, Flinders Sensitive Line rotte, Flinders Resistant Line rotte, monoamiene, depressiewe gedrag.

“If I have succeeded in my inquiries, more than others, I owe it less to any superior strength of mind, than to a habit of patient thinking.”

(Newton)

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"Sometimes you will never know the value of a moment, until it becomes a memory"

Dr. Seuss

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List of Abbreviations

Numerals and Symbols

5-HT	5-Hydroxytryptophan (Serotonin)
5-HT_x	5-Hydroxytryptophan (Serotonin) x – receptor subtype
5 – HIAA	5-Hydroxyindole acetic acid
$	Dollars
%	Percentage
°C	Degrees Celsius
6-OH DA	6-hydroxydopamine

A

ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotropic hormone
ACS	Acute coronary syndrome
ADHD	Attention-deficit/hyperactivity disorder
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AUC	Area under curve

B

BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor

C

cm	centimetre
CNS	Central nervous system
CPD	Chronic pre-synaptic depression
CREB	Cyclic adenosine monophosphate response element binding protein
CRH	Corticotrophin-releasing hormone

D

D_1	Dopamine receptor-type 1
D_2	Dopamine receptor-type 2
DA	Dopamine
DAT	Dopamine transporter
DFP	Diisopropyl fluorophosphate
DOPAC	3,4-Dihydroxyphenylacetic acid

E

EDTA	Ethylene-diaminetetraacetic acid

F

FDA	Food and Drug Administration
FRL	Flinders resistant line
FSL	Flinders sensitive line
FST	Forced swim test

G

g	grams

GLU	Glutamate
GP	Glycoprotein

H

h	Hour
HIV	Human Immunodeficiency Virus
HPA	Hypothalamic-pituitary-adrenal *axis*
HPLC	High performance liquid chromatography
HPLC-EC	High performance liquid chromatography system with electrochemical detection
HVA	4-hydroxy-3-methoxyphenylacetic acid

I

ICSS	Intra-cranial self-stimulation
IFN-γ	Interferon-gamma
IL-6	Interleukin-6

M

MAO	Monoamine oxidase
MAO-B	Monoamine oxidase type B
MAT's	Monoamine transports
MDD	Major depressive disorder
MDMA	Methylenedioxymethamphetamine
METH	Methamphetamine
METH-HCl	Methamphetamine hydrochloride
mg\kg	milligrams per kilogram
mm	millimetre

N

nAChRs	Nicotinic acetylcholine receptors
NE	Norepinephrine
NET	Norepinephrine transporter
nORT	Novel object recognition test
NPY	Neuropeptide-y
NWU	North-West University

O

OFT	Open field test
OTC	Over-the-counter

P

PCDDP	Pre-clinical drug development platform
PFC	Pre-frontal cortex
PnD	Postnatal day

R

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute

S

SA	South Africa
SASBCP	South African Society for Basic and Clinical Pharmacology
SC	Subcutaneous
SERT	Serotonin (5-HT) transporter

SSRE	Selective serotonin reuptake enhancer
SIT	Social interaction
SIR	Social isolation rearing
SNRI's	Selective norepinephrine reuptake inhibitors
SNP's	Single-nucleotide polymorphisms
SOD	Superoxide dismutase
SSRI's	Selective serotonin reuptake inhibitors
STD	Sexually transmitted disease

T

TNF-α	Tumour necrosis factor-alpha

U

U.K	United Kingdom
USA	United States of America
U.S	United States

V

VEGF	Vascular endothelial growth factor
VEH	Vehicle
VMAT-2	Vesicular monoamine transporter-2

W

WCP	World congress of basic and clinical pharmacology

Declaration

I, Moné Mouton, declare that the research proposal, planning of the experimental work, laboratory work, literature review, scoring of the behavioural study videos, data capturing and interpretation as well as preparing and writing the dissertation was conducted by myself, under the guidance of my supervisor, Prof C.B. Brink and co-supervisor, Prof B.H. Harvey.

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Moné Mouton (Student) Date

As supervisors to the study we confirm that the above declaration by Miss M Mouton is true.

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Prof C.B. Brink (Supervisor) Date

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Prof B.H. Harvey (Co-supervisor) Date

Chapter 1 - Introduction

This chapter describes (1) the approach and layout of the dissertation (the format of the dissertation), (2) the problem statement (a cryptic literature review that is discussed in detail in Chapter 2), (3) the objectives of the study, (4) the experimental design and approach of the study (i.e. the study layout), and (5) the expected results.

1.1 Dissertation format: approach and layout

This dissertation is presented in an article format. The most significant results of the study are prepared as an article to be submitted for publication in a peer review scientific journal (see Chapter 3). The following is an outline of the dissertation, serving as a guide to the reader:

- **Chapter 1**

  Introduction
  - Problem statement, study objectives, study layout and expected results.

- **Chapter 2**

  Literature review

- **Chapter 3**

  Scientific article
  - Depicting the behavioural test results which were most significant.

- **Chapter 4**

  Summary, final conclusions and recommendations (for the study as a whole, including the scientific article (Chapter 3) and the additional and supplementary results (Addendum B)).

- **Addendum A**

  Additional/detailed materials and methods

- **Addendum B**

  Additional and supplementary results and discussion
  - Depicting the results from the neurochemical analyses.

- **Addendum C**

  Instructions to the author (The International Journal of Developmental Neuroscience)

- **Addendum D**

  Congress contribution & young scientist award

- **Addendum E**

  References

1.2 Problem statement

Methamphetamine (METH) has been described as one of the most abused drugs in the United States of America (USA) (Stomberg & Sharma, 2012). A government survey reported that more than 10 million people have abused METH in the USA, while a 2005 figure suggests that the estimated total economic cost of METH abuse to be approximately $23.4 billion (Stomberg & Sharma, 2012). In addition, the Western Cape Province of South Africa has experienced a dramatic increase in the demand for treatment of drug abuse, such as for overdosing or treatment of addiction to cannabis (dagga), methaqualone (Mandrax® or Quaalude®), cocaine, heroin and METH (Nyabadza & Hove-Musekwa, 2010). In South Africa METH is often sold in drinking straws and costs as little as R30 per straw – sufficient for one dose (The Lancet, 2008). A 2006 study indicated that medical treatment related to METH abuse doubled during the period of 1992 to 2004 (Stomberg & Sharma, 2012). In fact, METH is the second most popular illicit drug world-wide (Cruickshank & Dyer, 2009). Thus, METH abuse is a fast growing and serious problem that some classify as an epidemic (Nyabadza & Hove-Musekwa, 2010). Attributing to this epidemic is the fact that METH is simple to synthesize from inexpensive and readily available materials, including over the counter drugs. This has contributed to easy and wide access to the drug, so that its abuse has increased globally. It is estimated that more than 35 million people are regularly abusing METH world-wide (Vos *et al.,* 2010).

In particular, the euphoric effects of this drug render it highly addictive and popular despite the risks and deleterious consequences of its abuse. An increase in energy levels and a

sense of happiness and weight loss are some of the sought after effects (Vearrier *et al*., 2012). However, these effects are commonly accompanied with a range of severe acute side-effects that can be life-threatening and fatal, for example arrhythmia, acute myocardial infarction, cardiomyopathy and coronary heart disease (Cruickshank & Dyer, 2009). Some coinciding consequences are irreversible, for example the contraction of HIV/AIDS. Although acute psychiatric effects such as psychosis, as well as withdrawal symptoms, may be deleterious, of most concern are the adverse effects following chronic abuse such as irreversible neurological damage and the increased risk of developing an anxiety disorder, depression and psychosis.

Depressive symptoms can be severe and debilitating to the user during and after withdrawal. Although depression is not uncommon under drug-dependent patients, METH produces a unique pseudodepressive state due to its ability to directly affect monoamine regulation. The symptoms of this state include anhedonia, fatigue, sleep abnormalities, loss of appetite, lack of motivation, irritability and poor concentration, so that this state relates to many of the characteristics of major depressive disorder (MDD) (McKetin *et al.,* 2011).

Since METH-induced depression is one of the key symptoms that require treatment during the withdrawal phase as well as later on in life, this study focuses specifically on the development of depressive-like behaviour following chronic (16 days) administration of METH or vehicle during pre-adolescence. An escalating dose regimen for METH was applied in order to mimic METH abuse behaviour, while pre-adolescent exposure was selected as this is the developmental period when METH is most often abused in humans. In addition, the study has investigated biomarkers serving as cues regarding the neurobiological basis that underlies the observed behavioural deficits. The study, furthermore, employed Flinders Sensitive Line (FSL) rats that are genetically predisposed towards greater stress sensitivity (regarded as a translational model of depression), as well as Flinders Resistant Line (FRL) rats that are regarded as the normal controls for the FSL rat. These animals will be used to investigate the role of genetic predisposition in the development of long-term side-effects following chronic METH administration. Depressive-like behaviours evaluated in this study included the novel object recognition test (nORT) to evaluate cognitive functioning (memory) , the Digiscan animal activity monitor to evaluate spontaneous locomotor activity of the animals, the social interaction test (SIT) to evaluate anxiety-like behaviour, and the forced swim test (FST) to evaluate despair-related depressive-like behaviour.

METH significantly increases monoamines in the central nervous system (CNS). It affects multiple neurotransmitter systems including the dopaminergic system, the serotonergic

system, the noradrenergic system as well as the glutamatergic systems. As a result increases in cytoplasmic concentrations of dopamine (DA) and serotonin (5-HT), as well as of norepinephrine (NE), histamine and adrenaline, are evident in humans (Karila *et al.,* 2009) and which has been advocated as mediating the addictive and behavioural affect characteristic of the drug. METH produces these effects by blocking the vesicular monoamines transporter 2 (VMAT2) intracellularly, by decreasing expression of the dopamine transporter (DAT) on the surface of the cell, by inhibiting the activity of monoamine oxidase and by increasing expression of tyrosine hydroxylase (Karila *et al.,* 2009). The monoamine hypothesis of depression suggests that depression is caused by impaired monoaminergic function in the brain (Krishnan & Nestler, 2008). The current study has therefore investigated the neurochemical changes in the frontal cortex following chronic METH exposure, focusing on DA, 5-HT and NE, as well as their metabolites. Neurochemical changes may be linked to depressive-like symptoms observed in METH abusing patients.

1.3 Study objectives

The primary objective of this study was to investigate the effect of early-life administration of METH to stress-sensitive (Flinders Sensitive Line - FSL) and control (Flinders Resistant Line - FRL) rats on depressive-like behaviour and regional brain monoamine levels later in life. The study implemented a sixteen-day period for administration of METH or a vehicle control from postnatal day 19 (PnD19) to postnatal day 34 (PnD34). The latter developmental stage corresponds to pre-adolescence in the rat when neurological development are similar to that seen in human adolescents, and represents the stage when drug abuse is most common in humans. The study then investigated whether early-life administration of METH would cause depressive-like behaviours directly after the injection period (immediate drug effects before withdrawal on post-natal day 35, i.e. PnD35) or later in life (after the withdrawal period in early adulthood on PnD60). The behavioural effects were assessed in a battery of tests on PnD35 or PnD60, followed by decapitation and brain dissection to assess altered neurochemistry.

The study specifically aimed to assess:

- Depressive-like behaviour in the forced-swim test (FST);
- Social interactive behaviour in the social interaction test (SIT);
- Self-directed behaviour in the social interaction test (SIT);
- Cognitive function in the novel object recognition test (NORT);
- Locomotor activity in the Digiscan (AccuScan) apparatus;

- A possible link between the observed behavioural changes in the animals and monoamine levels as well as their metabolites in the frontal cortex.

1.4 Study layout

This study, including all animal protocols, were approved by the Animcare Animal Research Ethics Committee of the North-West University (ethics approval no. NWU-000105-11-S5). All experiments conformed to the guidelines of the National Institutes of Health for the care and use of laboratory animals. The handling of animals was performed in accordance with the guidelines for the use of animals in experimental work at the North-West University. All animals were maintained according to a code of ethics in research, training and testing of drugs in South Africa and complied with national legislation.

- Animals: Male Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats were used in the study. The study included one early-life developmental stage of the rat, PnD19 to PnD34 ("pre-adolescence"). The pups were randomly divided into 8 different treatment groups. The groups contained between 10 and 16 rats per group as depicted in Figure 1-1 below.

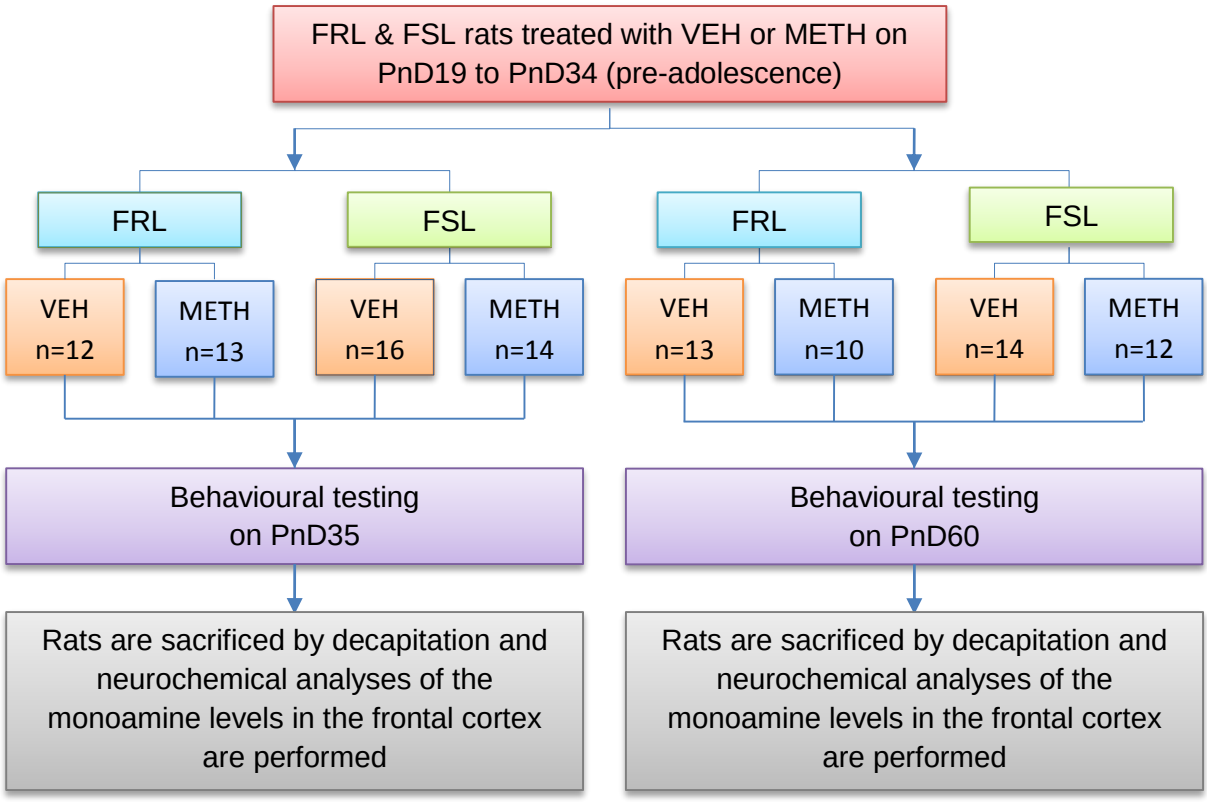

Figure 1-1: Study layout of the various treatment groups, differing regarding the rat line, the treatment received and the postnatal day of behavioural testing. The number of animals per treatment group is also indicated. FSL = Flinders Sensitive Line rat; FRL = Flinders Resistant Line rat; VEH = vehicle control; METH = methamphetamine HCL.

- Drug treatment: Chronic dosing of METH and saline was performed twice daily at 9:00 and 15:00 for 16 consecutive days. The METH-treated animals received a sub-cutaneous (SC) escalating dose regimen during the 16 day period, with twice daily doses escalating in increments of 0.2 mg/kg from 0.2 mg/kg to 6.0 mg/kg (Addendum A, Table A-1). METH administration was performed according to the body weight of the animals on that particular day, thus the animals were weighed before injection and thereafter the specific dose was calculated per individual rat. Saline-treated animals received a fixed dose of 0.2ml saline SC twice daily.

- Behavioural Testing: After the 16-day period of METH or saline administration, the study investigated the behavioural effects of such treatment, either directly after the injection period on PnD35, or later in life after drug wash-out (withdrawal) on PnD60. The following behavioural tests were implemented in the current study: the novel object recognition test (nORT) to evaluate cognitive functioning (memory), evaluation of spontaneous locomotor activity, the social interaction test (SIT) to evaluate anxiety-like behaviour and the forced swim test (FST) to evaluate depressive-like behaviour.

- Neurochemical analysis: The morning after behavioural testing concluded the rats were sacrificed by decapitation without any prior use of an anaesthetic agent. Whole brains were removed and the frontal cortex was dissected out. Monoamine levels were determined in the frontal cortex using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC).

1.5 Expected Results

The working hypothesis of this study is that chronic treatment with METH will induce depressive-like behaviours with corresponding neurochemical disruptions in key brain regions involved in depression. In addition, we expect that this study will improve our understanding of the neurobehavioural dysfunction brought about by early-life exposure to METH and enhance our understanding of the role of dysfunctional monoaminergic neurotransmission in the mechanism of how METH abuse may precipitate or worsen depressive-like symptoms. The current study will also produce a working hypothesis for future treatment options and direct further investigations and studies.

Chapter 2 - Literature review

2.1. Introduction to methamphetamine

Methamphetamine (METH) is a derivative of amphetamine and a synthetic psychostimulant. Although it has been registered with the U.S Food and Drug Administration (FDA) for the treatment of attention deficit disorder (Tar *et al.,* 2014), it is mostly abused for recreational purposes and presents with highly addictive properties. The ease with which METH is synthesized and produced from inexpensive and readily available materials, has increased worldwide. It is estimated that more than 35 million people are regularly abusing METH (Vos *et al.,* 2010; Mehrjerdi *et al*., 2014).

It is believed that amphetamine was first made in Germany in 1887, however the synthesis of METH from ephedrine by a Japanese scientist dates back to 1893. Then, in 1919 the first crystalline form was synthesised (McKellar, 2005). The crystalline form was readily soluble in water which provided an easy mode for injection. METH was used for the treatment of asthma as an inhalable spray in 1932. Soon thereafter, practitioners recognised the stimulating effects of the drug that lead to prescribing METH for narcolepsy and attention deficit hyperactivity disorder (ADHD) (Stedham, 2007). METH was also used in the 2nd world war for its stimulant properties by soldiers, rendering them courageous with enhanced endurance (Stedham, 2007; McKellar, 2005). Japanese Kamikaze pilots also received the drug before suicide missions. After the war ended, the drug supplies reserved for military use were made available to the general public. Thereafter, the number of people abusing METH escalated profusely. METH became a popular drug during the 1940s and 1950s, prescribed for a variety of indications which included depression as well as weight loss (Vearrier *et al*., 2012). METH was also used as a stimulant by people who were required to stay awake and concentrate for long periods at a time, for example students and truck drivers. Thus, the drug became freely available to all. Extensive revision and deviation of typical METH use during the 1960s and increasing awareness of the adverse health effects associated with METH, led to the withdrawal of most of the indications for licit METH use. This resulted in a decline in legal production of the drug. Consequently, the illicit manufacture of METH increased to meet the demand for the drug and augmented the abuse of the drug. During this time users preferred to use METH rather than cocaine, as cocaine was very expensive.

When it is in its crystalline form, the drug is colloquially referred to as crystal meth, ice, tina, tik, crank, glass or speed (McKellar, 2005). METH is a white/clear chunky crystal powder

that resembles ice and is odourless and bitter-tasting. It readily dissolves in water or alcohol and is administered orally, intra-nasally, by needle injection, by smoking (NIDA InfoFActs, 2010), swallowed, or inserted into the anus or urethra. Intermittent administration of METH and other psychostimulants has been shown to result in a progressive increase in psychomotor activation, a phenomenon referred to as behavioural sensitisation (Schutová *et al.,* 2009).

As a stimulant, METH causes an array of adverse effects, including the induction of a state of agitation and violence after the initial rush (Herman-Stahl *et al.,* 2007). METH is also associated with the transmission of HIV/AIDS, in particular since its psychotropic effects, including enhanced sexual desire, renders the abuser more vulnerable to risky sexual behaviour and thus to HIV infection (Vos *et al*., 2010). Pharmacologically, amphetamines have been shown to increase the activity of catecholaminergic neurotransmission, in particular of norepinephrine and dopamine (Dipiro *et al.,* 2011). METH's ability to release dopamine rapidly in specific regions of the brain is responsible for the resulting intense euphoria. The longer duration of euphoric effects of METH has attracted many users away from cocaine in favour of METH (Dipiro *et al.,* 2011). Continued use may result in severe weight loss, dermatological decay, uncontrollable rage, paranoia and depression. Levels of stress hormones, including cortisol and adrenocorticotropic hormone, are increased by over two hundred percent (>200%) following administration of METH. These levels may remain elevated for hours after exposure (Panenka *et al.,* 2012).

The abuse of METH and other amphetamine derivatives has developed into an epidemic in many countries (Mehrjerdi *et al.,* 2014) including South Africa (Cruickshank & Dyer, 2009). In fact, METH is the second most popular illicit drug world-wide (Cruickshank & Dyer, 2009; Bujarski *et al.,* 2014). Thus, although METH has been a common research subject for many years, the study of METH is still essential and further investigation into the mechanisms of how METH abuse causes depressive symptoms is required.

2.2. Epidemiology of METH

METH is said to be one of the most abused drugs in the United States of America (USA). A government survey reported that the estimated total economic cost of METH abuse was around $23.4 billion in 2005 (Stomberg & Sharma, 2012), whereas a 2006 study reported that METH abuse doubled during 1992 to 2004. By 2012 more than 10 million people have abused METH in the USA (Stomberg & Sharma, 2012). Moreover, a study that was conducted in Cape Town, South Africa, investigated the association between substance abuse and sexual activities among adolescents, and specifically the relation between METH

and risky sexual behaviours. The Western Cape Province of South Africa has experienced a dramatic increase in the demand for treatment of drug abuse, such as for overdosing or treatment of addiction to cannabis (dagga), methaqualone (Mandrax® or Quaalude®), cocaine, heroin and METH (Nyabadza & Hove-Musekwa, 2010). A very recent study estimated that 51 million people are abusing METH worldwide (Li *et al.,* 2014). This expanding global market of METH is fed by an increase in underground manufacturing of METH. Increases in the number of laboratories as well as their size and sophistication contribute to this rapid growth (Dipiro *et al.,* 2011).

It has been commonly reported that substance abusers are mostly among young adults (pre-adolescence), especially between the ages of 18 to 25 years (Cohen, 2014). However, METH abuse among teenagers appears to have dropped significantly in the United States of America in recent years, according to the U.S Department of Health and Human Services (NIDA InfoFacts, 2010). Subgroups of people who are especially prone to METH abuse include adolescents, sex workers, gay men and gang members (Lasco, 2014). People who live in rural areas are also more likely to abuse drugs than people in urban areas (Grant *et al.,* 2012; Woodall & Boeri, 2014). However, the abuse of drugs has infiltrated its way into the mainstream of cultures in many countries and is also common under celebrities. METH has also been coined a “Club-Drug” due to the fact that it is associated with the rave and club scene (Kelly *et al.,* 2006). In addition, METH and alcohol are often used in combination (Kucerova *et al.,* 2011), especially in a binge drinking pattern, whereas co-use of these substances may cause an exacerbation of adverse effects (Bujarski *et al.,* 2014). Not surprisingly the epidemiology of alcohol abuse is closely related to that of METH abuse.

Specific indicators have been used to determine the severity of stimulant abuse including treatment admissions, emergency room visits and even seizures as a result of drug overdose (Herman-Stahl *et al.,* 2007). Statistics indicate that 45% of all patients admitted for the treatment of METH abuse in 2003 were woman (Otero *et al*., 2006). This is particularly worrisome since women are in most cases the caretakers of children, thereby making a lifelong impression on their children. It has also been found that a third of METH abusers have been sexually and/or physically abused from a young age. Additionally, childhood trauma has been shown to increase the probability of psychosis later in life and findings have suggested that such an early-life ordeal may be a predictor for enhanced susceptibility to drug abuse (Ding *et al.,* 2014). Thus, the positive correlation between childhood abuse and drug abuse later in life is well recognised (Otero *et al*., 2006).

METH abuse is also associated with many drug-related crimes (Stomberg & Sharma, 2012). It has been reported that the likelihood of METH abuse was increased among those

individuals who participated in antisocial behaviours or criminal acts that included stealing, aggressive actions towards another person, previous arrests and binge drinking (Herman-Stahl *et al.,* 2007). Attributes, such as psychological distress, sensation seeking and attention are also commonly found under METH abusers. Interestingly, religiousness is relatively uncommon amongst METH abusers (Herman-Stahl *et al.,* 2007).

METH dependence causes significant agony, so much so that individuals abusing the drug experience negative social and emotional consequences, including the loss of family relationships, the inability to participate in educational and work activities as well as the involvement in criminal activities. However, these severe consequences do not seem to invoke cessation of the drug (Gowin *et al.,* 2013).

2.3. The neurobiology of depression

The prevalence of depression related to drug addiction is high (Kucerova *et al.,* 2011), and psychiatric illness such as depression has been clearly associated with METH abuse (Sutcliffe *et al.,* 2009; Bujarski *et al.,* 2014). METH abuse is also linked with schizophrenia (METH psychosis) that may present with depressive symptoms. In this regard, the negative symptoms of schizophrenia include social withdrawal, lack of energy and motivation, similar to those found in depression. Depressive symptoms frequently recounted by METH abusers may be either (1) related to pre-existing depressive symptoms or (2) to METH-induced adverse effects. The majority of METH users report a lifetime history of depression and over a third report a lifetime diagnosis of depression (Sutcliffe *et al.,* 2009). Depression is commonly believed to result from molecular and cellular abnormalities that relate to genetic and environmental factors (Duman & Voleti, 2012), while METH abuse and its subsequent adverse psychosocial effects presents as a significant environmental stressor.

Long-term METH use has also been associated with more severe psychiatric symptoms including paranoia, hallucinations and delusions. These findings may be attributed to a substantial reduction in dopamine transporter density in the brain (Smith *et al.,* 2012). Thus, an understanding of both the short- and long-term effects of METH on striatal dopaminergic markers is important. METH causes an increase in synaptic dopamine release, suggesting that long-term changes in dopamine signalling might underlie chronic pre-synaptic depression (CPD) (Welberg, 2008). Although many drugs with addictive properties exhibit acute increases in synaptic dopamine, METH and other amphetamines do so by inhibiting reuptake.

Studies have indicated that cessation of METH (i.e. withdrawal) leads to a reduction in depressive symptoms, which is independent of any specific treatment. This has been demonstrated in diverse samples where the study population included participants that differ in age, race and gender. In contrast, however, cessation of METH use is also associated with symptoms of depression as depression is a major component of the withdrawal syndrome (Sutcliffe *et al*., 2009). It has been reported that exposure to METH produces long-lasting depression of dopamine release at corticostriatal terminals, related to the METH-mediated release of dopamine. However, when METH is re-administered following cessation, this symptom is reversed (Bamford *et al.,* 2008). The latter study also indicated that METH-induced chronic presynaptic depression (CPD) is independent of long-term alterations in synaptic dopamine release, but rather due to changes in dopamine (D_1) and cholinergic receptors. Currently the literature is ambiguous on whether experiencing depressive symptoms promotes METH abuse or whether depression results from or is enhanced by METH use, or a bidirectional result exists. Results of studies conducted to answer this question are inconsistent (Sutcliffe *et al.,* 2009). However, is has been suggested that an integrated treatment approach that considers both the patient's mental health and cessation of drug abuse may be successful in treating dependence without resulting in depression (Sutcliffe *et al.,* 2009). It is important to keep in mind that the majority of studies regarding METH-associated depression have been conducted within the context of treatment trials and thus could represent a smaller and less diverse population of METH abusers than would normally be found in a community sample (Sutcliffe *et al.,* 2009).

As mentioned above, neurobiological alterations underlie depression and that the origin thereof can be described through a model that includes both environmental and biological causes. Thus, a number of hypotheses exist concerning the neurological basis of depression and are discussed below.

2.3.1. Hypotheses of depression

2.3.1.1. The monoamine hypothesis

The most well-described and supported hypothesis of depression is the *monoamine hypothesis (biogenic amine hypothesis)* that suggests that depression is caused by dysregulation of monoaminergic neurotransmission, especially in the limbic regions of the brain (Krishnan & Nestler, 2008; Sapolsky, 2000). This hypothesis resulted from observations that were made during the 1950's where researchers found that the antihypertensive drug reserpine caused a depletion of neuronal storage granules of DA, 5-HT and NE and consequently resulted in depressive symptoms (Dipiro *et al.,* 2011).

Reduced monoamine metabolite levels have been found in cerebrospinal fluid of depressed individuals and these findings are in line with the increase in monaminergic signaling that is observed with effective antidepressant treatment (Ansorge *et al.,* 2007). Accordingly, the measurement of monoamine levels in the frontal cortex is of particular importance in the current study. It is however now evident that no single neurotransmitter theory of depression is suitable (Dipiro *et al.,* 2011). Serotonergic and noradrenergic systems are involved in anti-depressant responses that are consistent with the rationale of the postsynaptic alteration theory where β-adrenergic receptor down-regulation is vital for achieving an antidepressant effect (Dipiro *et al.,* 2011). Studies have also demonstrated that the function of the serotonin transporter (SERT) plays an important role in the pathophysiology of depression. Hence, selective serotonin reuptake inhibitors (SSRI's) which blocks serotonin reuptake via the SERT and consequently increases serotonergic neurotransmission are currently first-line treatments for depression (Ansorge *et al.,* 2007). Although the monoamine hypothesis mainly focuses on 5-HT and NE, studies have found that agents that increase dopaminergic transmission are effective antidepressants. An increase in DA transmission in the mesolimbic pathway has been suggested to account for the antidepressant effects observed (Dipiro *et al.,* 2011). All current antidepressant thus adhere to this hypothesis by increasing monoamines in the brain. It is important to note that most, if not all, new hypotheses of depression eventually impact or relate to the monoamine hypothesis and therefor the biogenic amine hypothesis will always remain relevant. Some limitations to this hypothesis have however been identified: depressed patients respond differently to the same antidepressant, drugs like cocaine and amphetamines that increase monoaminergic activity in the brain are not clinically effective antidepressants, and an increase in synaptic monoamine levels is seen within hours after of the administration of antidepressants treatment, yet, antidepressant effects are only seen after continuous administration for 3-6 weeks (Baldessarini, 1989; Racagni & Popoli, 2008).

Recent studies have suggested that emotional behaviour is controlled through a delicate balance between NE and 5-HT levels and thus *the permissive hypothesis* (modified from the monoamine hypothesis) was developed (Hilty *et al.,* 2006). According to this hypothesis manic and depressive episodes are characterised by a decreased central 5-HT function (Spencer, 1977). Serotonergic systems are responsible for inhibiting a range of other neurotransmitter functions and consequently mood disorders result from the removal of this serotonin inhibition (Hilty *et al.,* 2006). Thus, deficits in 5-HT can cause NE levels to fall below normal ranges which then results in depression, while manic episodes are observed if NE levels are increased (Hilty *et al.,* 2006). 5-HT thus exerts a permissive role in how changes in NE will affect mood, with changes in 5-HT being a prerequisite before altered NE

levels impact mood (Harvey, 1997). METH causes the rapid release of 5-HT and NE into the synapse and may thus disrupt the balance between these transmitters resulting in mood disorders such as depression on the one hand or mania or hypomania on the other.

2.3.1.2. The neuroplasticity hypothesis

The *neuroplasticity hypothesis* postulates that a decrease in neurotrophic factors (which regulate plasticity in the brain) renders the brain more susceptible to neuronal degeneration following stress, as observed in patients suffering from long-term MDD (Serafini, 2012). Depression is therefor associated with structural brain changes, especially of the hippocampus and frontal cortex, accompanied by deficits in neurotrophin support, for example brain derived neurotrophic factor or BDNF (Ansorge *et al.,* 2007). These deficits in neurotrophic factors together with anatomical changes as well as chronic stress associated with depression represent the central theory of the neuroplasticity hypothesis of depression. Several studies have indicated that reduced neurotrophic factor signalling in the brain may be associated with the pathophysiology of depression, where environmental and psychosocial stressors are causally related to the reduced expression of neurotrophic factors in the limbic structures of the brain (Ansorge *et al.,* 2007).

The most important neurotrophic factors are the transcription factor cyclic adenosine monophosphate response element binding protein (CREB) and BDNF both responsible for regulating neuronal growth and resilience (Charney *et al.,* 2004). Chronic stress and an associated increase in glucocorticoids, like cortisol, may result in disruptions of BDNF expression (Dipiro *et al.,* 2011). Reduced secretion of BDNF is associated with reduced hippocampal volumes, abnormal hippocampal function and poor memory observed in patients with depression. In addition, chronic stress in rodents similarly reduces neurotrophic factor levels, adult hippocampal neurogenesis and total hippocampal volume (Ansorge *et al.,* 2007). Thus the FSL rat model of depression also displays significantly decreased levels of BDNF and vascular endothelial growth factor (VEGF) as well as a reduction in neuronal and synapse numbers as a consequence (Overstreet & Wegener, 2013). Data also indicates that antidepressant therapy, especially SSRI's and selective noradrenaline reuptake inhibitors (SNRI's), can reverse attenuated BDNF levels in depressed individuals and relieve depressive symptoms, thus supportive of the neuroplasticity hypothesis (Atake *et al.,* 2014; Celikyurt *et al.,* 2012).

2.3.1.3. The cholinergic super-sensitivity hypothesis

The *cholinergic super-sensitivity hypothesis* postulates that depression and manic episodes are associated with hyper- and hypo-cholinergic states, respectively, which are associated

with, and may explain, corresponding increased and deceased noradrenergic neurotransmission (Dilsaver, 1986). This hypothesis first emerged when depressive-like symptoms were observed in humans with organophosphate poisoning. Organophosphates caused the inhibition of acetylcholinesterase (AChE) and consequently an increase in acetylcholine (ACh) levels (Dilsaver, 1986; Mineur & Piciotto, 2010). In addition it has been documented that when a centrally acting AChE inhibitor, physostigmine, is administered, depressive-like symptoms are induced in humans. Centrally acting anticholinesterases and cholinomimetics, via muscarinic receptors (Fritze *et al.,* 1995), produces symptoms such as dysphoria, psychomotor retardation, malaise and the inability to sleep (Dilsaver, 1986) and are similar to depressive symptoms. The FSL rat was selectively bred to display increased sensitivity to the centrally acting AChE inhibitor diisopropylfluorophosphonate (DFP) resulting in increased ACh levels in the brain. The FSL rats also display an increase in the number and function of high affinity nicotinic acetylcholine receptors (nAChRs) and consequently a predisposed cholinergic sensitivity (Mineur & Piciotto, 2010). Recently, clinical studies have found that centrally acting antimuscarinic agents, such as scopolamine are effective as an adjunctive treatment in treatment resistant depression (Furey & Drevets, 2006).

2.3.1.4. The hypothalamic-pituitary-adrenal-axis hypothesis

The *hypothalamic-pituitary-adrenal-axis hypothesis*, also well documented, postulates that chronic increased levels of corticosteroids lead to decreased biological neurogenesis resulting in toxic effects on the hippocampus, a key brain region involved in regulating the stress response (Oriaifo *et al.,* 2012; Holsboer, 2001). These pathological changes manifest in the form of cognitive deficits and other typical symptoms of major depressive disorder (MDD). Chronic stress causes an increase in adrenocorticotropic hormone (ACTH) release from the pituitary gland following up-regulated release of corticotrophin-releasing hormone (CRH) from the hypothalamus. The state of hypercortisolaemia is associated with deficits in normal feedback inhibition on the raised CRH which then remains elevated. This hypothalamic-pituitary-adrenal (HPA) axis dysregulation is related to depression (Oriaifo *et al.,* 2012). This hypothesis is particularly successful in explaining metabolic disorders typically associated with longstanding major depression. Several of the HPA axis receptor genes are associated with a number of metabolic irregularities. HPA axis activation may contribute to glucose intolerance, obesity as well as increased triglycerides and blood pressure (Gragnoli, 2014). Increased cortisol levels results in increased glycogenolysis, gluconeogenesis and free fatty acids. In addition, it reduces insulin secretion and increases insulin resistance. All of these effects lead to the development of type 2 diabetes mellitus

and metabolic syndrome (Gragnoli, 2014). Dysregulation of the HPA axis can also result in sympatho-adrenal hyperactivity and may result in an increase in vasoconstrictive tone, increased heart rate as well as platelet activation (Baune *et al.,* 2012) each of which has been implicated in cardiovascular disease. The HPA axis is also responsible for modulating sleep and dysregulation can disrupt circadian rhythms where sleep deprivation (insomnia) is associated with HPA activation. Therefore, investigation of the neurobiological changes observed during depression has focused on the HPA axis. The HPA axis serves as a marker for both stress response and a mediator of further pathophysiologic changes which includes the loss of neuronal plasticity (Oriaifo *et al.,* 2012). It is thus crucial that antidepressant drugs include actions on the HPA system, so that CRH receptor (type 1 and 2) antagonists are now being targeted as novel antidepressants (Oriaifo *et al.,* 2012).

2.3.1.5. The neurodevelopmental hypothesis

The neurodevelopment hypothesis is currently one of the leading theories of depression. Recent hypotheses recognised that genetic and adverse environmental factors (such as METH abuse) may increase susceptibility to depression (Ansorge *et al.,* 2007) and that investigating these factors during development may provide new insights on the basis and pathophysiology of depression.

It has been documented that parental depression may have negative effects on child development so that parental depression and/or anxiety must be recognised as a child's earliest adverse life event (Koehler, 2006). The effects of depression on foetal development during pregnancy can be transferred firstly by neurobiological substrates of depression such as pro-inflammatory cytokines and glucocorticoids (cortisol); secondly, indirect neuroendocrine processes such as hyperactivity of the HPA that may induce placental hyper-secretion of CRH, and thirdly dysphoric effects on foetal development indirectly through parental behaviour (Koehler, 2006). The above mentioned effects may affect numerous aspects of cognitive and psychosocial function in the foetus and usually persists beyond infancy. Emotional and behavioural deficits have been observed in preschool children that have been subjected to maternal stress. These deficits do not only occur when children are exposed to stress in the womb, but also exposure at a young age. An array of evidence thus demonstrates that early life adversity contributes significantly to the susceptibility to adulthood psychopathology, medical illnesses, as seen in Figure 2-1, (such as depression), maladaptive psychosocial functioning (Koehler, 2006) as well as altered hippocampal plasticity (Ansorge *et al.,* 2007). Thus, as METH abuse and its adverse psychological effects are a significant environmental stressor, especially during early adulthood or adolescence, this may introduce a significant risk for developing MDD in later

life. Moreover, METH abuse during pregnancy will constitute a psychosocial stressor that will place the child at significant risk for developing a mood and/or an anxiety disorder later in life.

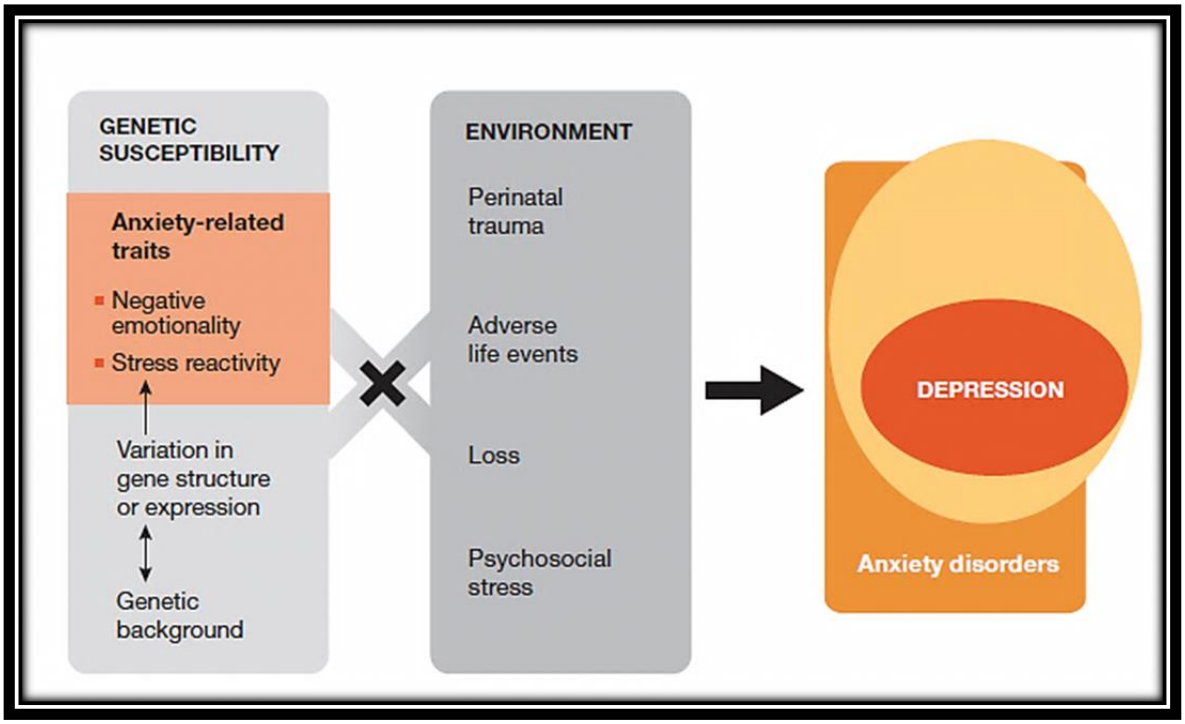

Figure 2-1: Vulnerability and co-morbidity of depression: neither genes nor environmental factors act alone (Adapted from Lesch, 2007).

2.3.1.6. The inflammatory hypothesis

During the 1990's evidence was found in support of immune activation in MDD, where elevated levels of pro-inflammatory cytokines (TNF-α and IL-6) and an increase in peripheral leukocytes, monocytes and T-cells were observed (Soskin *et al.,* 2012; Hashmi *et al.,* 2013). These findings gave rise to *the inflammation hypothesis* of depression (Soskin *et al.,* 2012). Pro-inflammatory cytokines may produce dramatic alterations in behaviour ("sickness behaviour") by acting on the brain. Symptoms include anhedonia, impaired concentration and sleep disturbances which strongly resemble the neurovegetative features of MDD. These pro-inflammatory cytokines may also induce indoleamine 2,3-dioxygenase (IDO), which then shifts tryptophan metabolism toward the production of kynurenine and quinolinic acid rather than 5-HT and thus decreasing 5-HT levels (Soskin *et al.,* 2012). Studies have documented a positive correlation between depression and a decreases in trypotophan, an increases in kynurenin and thus low levels of 5-HT (Soskin *et al.,* 2012). It has also been documented that administration of cytokines to rodent models is associated with depressive-like symptoms (Soskin *et al.,* 2012) and may be reversed through treatment with anti-

inflammatory cytokines such as interleukin-10 or a cytokine antagonist. In humans, biological stressors such as pathogens as well as psychological stress can activate the inflammatory response while such individuals have been found to develop depressive-like symptoms (Soskin *et al.,* 2012). Indeed, patients receiving interferon-α (an inducer of IDO) for the treatment of certain cancers often present with severe depressive-like symptoms (Raison *et al.,* 2005). Since METH activates the inflammatory response (Coelho-Santos *et al.,* 2012) this could explain why most METH abusers develop depressive-like symptoms.

2.4. Major depressive disorder (MDD)

Mental disorders contribute to the impediment of diseases in every country and about 14% of this global burden can be attributed to neuropsychiatric disorders (Tomlinson *et al.,* 2009). Major depressive disorder (MDD) is a mental disorder characterised by a pervasive and persistent low mood and self-esteem that is accompanied by loss of interest or pleasure in normally enjoyable activities. South Africa has lower rates of depression when compared to the USA, but higher rates than other African countries like Nigeria (Tomlinson *et al.,* 2009).

In order to meet the *Diagnostic and statistical manual of mental disorders*, 4th edition (DSM-IV) criteria for a major depressive episode, five or more symptoms of depression must have been present during the same 2-week period. MDD is defined as a chronic condition where the patient suffers from at least one of the core symptoms, as well as at least four of the following secondary symptoms listed below. Core symptoms: (i) lack of motivation and losing interest rapidly (ii) incapable of experiencing pleasure (anhedonia). Secondary symptoms: (i) loss of appetite, (ii) insomnia, (iii) motor retardation and/or agitation, (iv) sustained fatigue (v), a sense of worthlessness or the constant feeling of guilt, (vi) cognitive deficits, and (vii) suicidal thoughts (Yadid *et al.,* 2000). The severity of MDD is coded as mild (5–6 symptoms), moderate (7 symptoms) or severe (8 symptoms) (McKetin *et al.,* 2011; Hashmi *et al.,* 2013).

Physiological and biochemical characteristics that are often observed in these patients include chronic pain, high levels of plasma cortisol, super-sensitivity to cholinergic agonists and close relatives that also suffer from depressive disorders indicating a genetic factor. If left untreated, MDD can reduce adherence to drug treatment, increase the risk of relapse and elevate the risk of suicide.

2.5. Adverse effects of METH abuse

As a stimulant, METH increases vigour and alertness while decreasing appetite and fatigue. A tendency to sleep deprivation has also been reported (Stedham *et al.,* 2007). The effects of this powerful addictive stimulant can last from four to sixteen hours. Even in small

quantities, METH can result in many effects similar to those of other stimulants such as cocaine or other amphetamines. These effects include increased respiration, rapid heart rate, arrhythmias, hypertension and hyperthermia. It is reported that when METH is administered parentally it causes an immediate extremely pleasurable rush that continues for a few minutes, which is associated with a rapid development of tolerance (Stedham *et al.,* 2007). However, when METH is ingested orally or "snorted" (intra-nasal administration) it produces effects only within 15 to 20 minutes.

Both short- and long term effects on general health have been documented. For example, METH-induced caries, commonly known as "meth mouth", which is characteristic of dental decay and is more frequently observed in users that administer the drug via smoking (Dipiro *et al.,* 2011). Anxiety, confusion, insomnia, mood disturbances, violent behaviour and brain damage have also been observed. Chronic METH abusers can also display a number of psychotic features, including paranoia, visual and auditory hallucinations, and delusions (for example, the sensation of insects crawling under the skin). Emotional, physical and sexual abuse, as well as neglect, usually accompanies METH abuse. It is also vital to consider the indirect adverse effects that the use of METH has on individuals and the society. Acetone, red phosphorous, ethyl alcohol and lithium are all flammable ingredients that are used in METH cookers. As a result fires and explosions often occur during the preparation of administrations, causing severe burns (Dipiro *et al.,* 2011).

METH administration is normally followed by a "crash" when the drug is metabolised, causing a sense of depression. To avoid the latter, the user will administer a supplementary dose which is normally greater than the initial dose. This administration cycle can continue for a couple of days and results in a lack of food intake and a minimal amount of sleep. During this time the user may also experience increased paranoia and aggression (Stedham *et al.,* 2007).

2.5.1. Neurological and psychiatric effects

Chronic METH abuse is associated with neurochemical, psychiatric, and cognitive impairments (Kobeissy *et al.,* 2014) that persist long after the short-term effects of the drug have subsided. Images of the brain of METH-dependant individuals have indicated structural abnormalities of the brain, including grey-matter deficits, a decrease in the size of the hippocampus, white-matter hypertrophy, damage to the medial temporal lobe and enlargement of the striatum (Karila *et al.,* 2009). Decreases in striatal DA transporter (DAT) availability and a greater reduction in transporter density in the brain are associated with psychiatric, memory and motor deficits. These effects have also been reported in METH

abusers. In addition, impairments in learning, memory and information processing speed have been observed (Riddle *et al.,* 2006).

One of the most devastating adverse health effects of METH is intracranial haemorrhage, which is normally caused by METH-induced hypertension and tachycardia. METH is even more likely to cause intracranial haemorrhage than cocaine, due to its extended cardiovascular effects (Vearrier *et al*., 2012). Bleeding in the cerebrum, cerebellum, basal ganglia and brainstem have been reported and usually results in death. METH is also associated with ischemic stroke and seizures. These adverse effects can occur in the absence of chronic hypertension or cerebral vasculopathy and with or without a history of seizures (Vearrier *et al*., 2012). Long-term METH abuse also results in cognitive impairment, as evidence by a poor performance in memory tests and the inability to process information. METH abusers also have a shorter attention span and difficulty in abstract thinking. Cognitive impairment also seems to worsen when a frequent higher dose of METH is used. However, these patients do not display any impairment in apparent intelligence and verbal fluency (Vearrier *et al*., 2012).

METH-abusing patients have an increased risk of traumatic injuries resulting from violent behaviours. METH induces a state of agitation and violence after the initial rush (Herman-Stahl *et al.,* 2007). Many patients arrive at the emergency room with stab wounds and gunshot wounds as a result of drug-related violence. Attempted suicides, which may be associated with depression, are also more prevalent under METH abusers (Vearrier *et al*., 2012). These suicide attempts usually occur while the individual is intoxicated and involves deliberate overdose, lacerations to the arms and wrists and trauma resulting from jumping from extreme heights. Self-mutilation is also common under METH abusers and is usually recurrent (Vearrier *et al*., 2012). Domestic violence and altercations with the law are also more prevalent among METH abusers.

The question of why METH-dependant individuals make inappropriate, impulsive and risky decisions has been the subject of numerous investigations. Neuroimaging studies have been central to the search for an answer. METH dependant individuals constantly make decisions between losses and gains. In this regard, METH-dependence has been shown to reduce the activity of the bilateral rostral anterior cingulate cortex and to increase activation of the left posterior insula, both of which are involved in decision-making (Gowin *et al.,* 2013). Consequently, patients make decisions based on a lesser amount of information than healthy controls (Ermakova *et al.,* 2014). Thus, these findings add to the neurobiological explanation that, although the negative consequences of the drug are clear, individuals continue to use the drug because possible losses are ignored or seen as irrelevant or

manageable. The latter provides a plausible explanation to why cessation and undergoing treatment is so challenging for a METH-abusing patient. This data also demonstrates that neuroimaging can potentially provide some valuable insight as to new and innovative treatment options and the efficacy of drug treatment. Optimising treatment by targeting decision-making and the difference between instant gains or long-term losses can be beneficial (Gowin *et al.,* 2013). The latter treatment involves practising safe decision making with a counsellor by identifying safe versus risky decisions using a set work sheet that includes relevant scenarios (Lee *et al.,* 2007).

2.5.2. Neurotoxicity of METH

When considering the mechanism of how METH cases neurotoxicity, the differences between the acute versus chronic pharmacological effects of METH abuse are important. In addition, METH abusers are usually polysubstance abusers and may have pre-existing brain abnormalities (Panenka *et al.,* 2012). Thus, irregularities that are found when investigating the brain function of such an abuser may be as a result of a number of substances including cannabis (dagga), methaqualone (mandrax) or cocaine and not merely METH (Panenka *et al.,* 2012).

Amphetamines are weak monoamine oxidase B (MAO-B) inhibitors, thus limiting the oxidative deamination of monoamines and results in the accumulation of toxic metabolites such as 6-hydroxydopamine (6-OH DA) (Marek *et al.,* 1990). A reduction in metabolism also occurs due to a reduction in the reuptake of the drug, thus adding to its toxicity. Toxicity following the use of METH is a result of the release of catecholamines from the peripheral and central nervous system (CNS). Studies on the neurotoxicity of METH in monoaminergic terminals indicate that amphetamine toxicity involves the occurrence of at least three acute events: an increase in extracellular and intracellular DA, an increase in extracellular glutamate (GLU), and hyperthermia. The major molecular mechanisms by which these events induce their long-term adverse effects are via oxidative stress, excitotoxicity, and mitochondrial dysfunction (Yamamoto *et al.,* 2010).

Numerous studies have reported that damage to or loss of dopaminergic neurons occurs after exposure to METH (Panenka *et al.,* 2012), however, a smaller number of studies have reported actual cell death due to striatal apoptosis, characterised by the activation of cysteine proteases called caspases (Gold *et al.,* 2009). Current thinking is that DA signalling in the striatum of intense METH abusers may be impaired pre-synaptically by the reduction in the amounts of DA available for transmission, and post-synaptically by impairing second-messenger signalling mediated through adenylyl cyclase. These impairments may be

important in mediating the dysphoric effects of the drug (low dopamine) and the tolerance that occurs after repeated dosing (Panenka *et al.,* 2012). Evidence also suggests that continuous use of METH can cause degeneration of DA neurons, possibly because of the formation of an endogenous neurotoxin, 6-OH DA. In addition to low DA levels, exposure to METH has also been linked with neurotoxic effects on the central serotonergic systems. METH causes significant long-term reduction in markers of serotonergic terminals (Panenka *et al.,* 2012). However, the mechanism of METH-induced serotonergic toxicity is not as clear as for DA. Based on these studies, abusers of METH may have a greater risk of developing chronic neuropsychiatric disorders, which can continue for varying duration after discontinuation of the drug (Panenka *et al.,* 2012).

2.5.3. METH and CNS inflammation

Recent studies have reported the involvement of a neuro-inflammatory process that results in brain dysfunction following chronic METH use. In a recent study it was demonstrated that METH triggers a neuro-inflammatory response when administered as a single dose of 30 mg/kg (Coelho-Santos *et al.,* 2012). The pathology of neurodegenerative diseases involves neuro-inflammation that result in the release of cytotoxic mediators such as cytokines, redox active substances and excitotoxins from microglia that lead to neuronal damage (Tocharus *et al.,* 2010). Microglia cells are the primary immune cells that are sensitive to foreign stimuli. When these cells are activated by aversive stimuli they migrate to the site of injury. There they secrete a range of reactant factors that can cause damage to neuronal tissues i.e. pro-inflammatory cytokines and free radicals (Tocharus *et al.,* 2010). Cytokines that are released by microglial cells play an important role in the event of injury to the brain. Cytotoxic factors produced by microglia may add to the CNS deterioration seen in METH abusers (Tocharus *et al.,* 2010). In fact, researchers have established that glial cells are activated following METH exposure which contributes to METH-related neuropathology (Gonçalves *et al.,* 2010; Yamamoto & Raudensky, 2008). In addition, apoptosis of microglial cells occurs together with a decrease in cell proliferation (Coelho-Santos *et al.,* 2012). Furthermore, METH causes oxidative stress and the overproduction of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) (Tocharus *et al.,* 2010). In these studies (Tocharus *et al.,* 2010) pre-treatment with melatonin was found to neutralise METH-associated ROS and RNS production in microglia and might thus be considered as a therapeutic agent to protect the brain against METH-induced neurodegeneration. In support of this, in a recent study conducted in our laboratory, we found that the biological antioxidant, melatonin, is able to protect against oxidative stress and the adverse effects of oxidative stress on memory

(Mokoena *et al.,* 2014). Recent data confirms that chronic melatonin administration significantly improves stress-induced cognitive dysfunction (Mokoena *et al.,* 2014).

2.5.4. METH associated HIV/AIDS risk

METH abusers are more likely to contract a sexually transmitted disease (STD) and blood-borne diseases than non-users (Vearrier *et al.,* 2012; Maxwell, 2005 ; Mehrjerdi *et al.,* 2014), where sharing needles serves as the main vector for transmission of these diseases. The intoxicating effects of METH, regardless of how it is administered, can alter decision-making skills and can lead users to engage in dangerous and high risk behaviours, particularly risky sexual behaviours (Plüddemann *et al*., 2008; Desplats *et al.,* 2014; Urbina & Jones, 2004). Transmission of the human immunodeficiency virus (HIV), Hepatitis B and C and other infectious diseases can be transmitted through contaminated needles, syringes and other injection equipment that is used by more than one person (Mehrjerdi *et al.,* 2014). STD's including chlamydia, syphilis, and genital and oral gonorrhoea has been reported among METH-abusing gay men (Vearrier *et al.,* 2012).

The link between drug abuse and HIV infection is well known and the effect of METH on sexual behaviour is cause for concern. South Africa already has one of the highest HIV prevalence figures in the world (Plüddemann *et al*., 2008). A decrease in DA levels and neuron degeneration found post mortem in HIV-infected individuals suggests that HIV infection might damage nigrostriatal DA neurons (Yamamoto *et al*., 2010). In fact, it has been shown that HIV damages other brain areas as well, such as the prefrontal cortex, parietal cortex, nucleus accumbens and hippocampus. Such damage to the areas of the brain could make a HIV-infected METH abuser more vulnerable to METH toxicity, creating a vicious circle (Yamamoto *et al*., 2010). The combined effects of HIV infection and chronic METH exposure produce neuronal damage and inflammation (as described in § 2.5.3). METH and HIV both cause an increase in the permeability of the blood brain barrier (BBB), resulting from damaged tight junction proteins of the BBB (Ramirez *et al.,* 2009). In HIV-infected METH users, the METH-induced increase in BBB permeability might aid the transport of HIV-infected leukocytes across the BBB into the brain (Ramirez *et al.,* 2009). METH and the HIV protein GP120 greatly increase transendothelial migration of immunocompetent cells across the BBB. Consequently, METH may contribute to HIV-induced BBB breakdown by causing the release and/or activation of matrix metalloproteinases (Yamamoto *et al.,* 2010).

In further support of the destructive synergism between HIV and METH, studies of HIV positive METH abusers indicate that HIV causes greater neuronal injury and cognitive

impairment in individuals in this group compared with HIV-positive people who do not use the drug (NIDA, InfoFActs, 2010). In addition, METH has been shown to enhance HIV infection of macrophages, the primary target of the virus (Yamamoto *et al*., 2010). Consequently, METH abuse may also exacerbate the progression and prognosis of HIV/AIDS and its effects.

2.5.5. Cardiovascular and hepatic effects

One of the most common symptoms associated with METH abuse is chest pains, suggesting acute coronary syndrome (ACS). Other cardiac complications due to METH abuse reportedly include arrhythmia (due to myocardial ischemia) and acute myocardial infarction, and chronic METH abuse may result in cardiomyopathy and coronary heart disease (Cruickshank & Dyer, 2009). The mechanism by which METH induces these cardiac complications includes accelerated atherosclerosis, increased stimulation of cardiac noradrenergic receptors, hypercoagulability, and epicardial coronary artery spasms (Vearrier *et al.,* 2012). METH-induced anxiety, hypertension and tachycardia may cause death due to ruptured berry aneurysm (Vearrier *et al.,* 2012). The latter, also referred to as vascular "ballooning", is a cerebrovascular disorder where the wall of a cerebral artery or vein dilates in a specific location where the wall is weak.

Drug-induced organ damage to the liver, kidneys and pancreas, associated with renal failure, pancreatitis and hepatic necrosis has been reported (Vearrier *et al.,* 2012). METH abusers are also at greater risk of contracting hepatitis (hepatitis A, B and C) due to unsafe sexual behaviours as well as the reuse of contaminated needles. Hepatitis C infection is usually seen in abusers who smoke the drug. In addition, METH may cause acute liver injury associated with hepatic necrosis. METH can also augment the hepatic toxicity of other drugs (Vearrier *et al.,* 2012).

2.5.6. Oral and Dermatological Effects

METH-induced caries, commonly known as "meth mouth", is characteristic of dental decay frequently observed in users that administer the drug via smoking (Dipiro *et al.,* 2011) (Figure 2-2). "Meth mouth" is one of the most publicised effects of METH abuse and is used to increase awareness of drug abuse. There are multiple mechanisms that cause tooth decay in METH abusers, including sympathetic overstimulation and bruxism as well as the lack of dental hygiene and malnutrition. The consumption of soft drinks that contain large amounts of sugar to relief xerostomia (dry mouth) also contributes to poor oral hygiene (Vearrier *et al*., 2012).

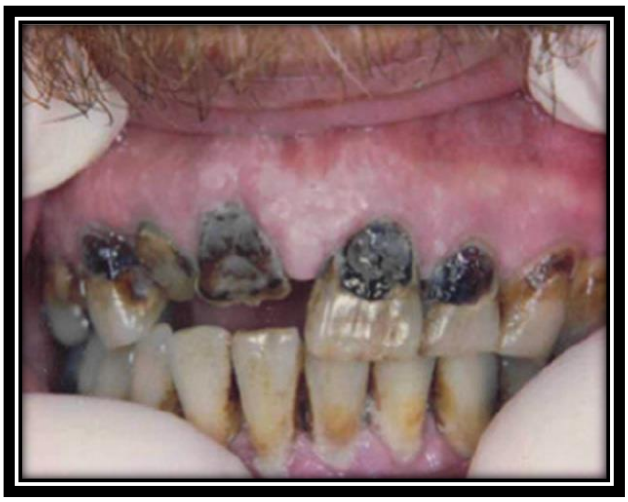

Figure 2-2: "Meth Mouth". Severe dental caries and deterioration of teeth due to methamphetamine abuse (Vearrier *et al.,* 2012).

As mentioned earlier, chronic METH abusers can also display a number of psychotic features, including paranoia, visual and auditory hallucinations, and delusions, notably the sensation of insects crawling under the skin. The latter may be attributed to a substantial reduction in DAT density in the brain (Smith *et al.,* 2012). METH abusers are also inclined to skin-piking, which leaves lesions on the face and extremities. Due to unhygienic conditions these lesions may become infected, resulting in cellulitis and cutaneous abscesses (Vearrier *et al*., 2012). Continuous injections in the same area of the body may result in soft tissue infections and apoptosis. In addition, the production of METH in "home-made" METH cookers frequently results in fires and explosions, which may lead to severe heat and acid burns of the skin.

2.5.7. METH dependence and treatment

The euphoric and addictive properties of METH causes users to abuse the drug with an increase in frequency and dose, even though it might not have been their original intention. Addiction is associated with pathophysiological changes in certain brain regions, notably the hippocampus, ventral tegmental area, nucleus accumbens and pre-frontal cortex (Kobeissy *et al.,* 2014). Drug craving and relapse (addiction-associated behaviours) result from synaptic changes that continue long after withdrawal (Bamford *et al.,* 2008). Persistent use of METH can lead to tolerance and thus a continuous increase in dose will be required to achieve the desired effects. This will then, in turn, lead to the typical drug-seeking behaviour associated with METH abuse. METH dependence has become a worldwide health problem and results in many medical conditions, psychiatric illnesses, cognitive abnormalities, socioeconomic difficulties as well as legal problems (Karila *et al.,* 2009). The timing and

intensity of the euphoria experienced by the user is dependent partly on the method of administration. For example, when smoked or injected the effects occur almost instantaneously, however the time to onset may be delayed for up to five minutes when "snorted" and twenty minutes when swallowed (Dipiro *et al.,* 2011).

Women often use METH to induce weight loss. However, these effects are short-term. As the drug is used, the body builds up a tolerance to the drug and thus the amount of weight loss declines and ceases completely at around six weeks after initiation. In addition, the weight loss is regained as soon as a person stops using METH. Due to the latter and the fact that this drug is highly addictive, METH is not advocated for weight loss (Dipiro *et al.,* 2011).

The treatment of addiction is a complex process and requires multiple approaches where pharmacological and behavioural therapies are often combined to provide a complete therapeutic approach. The treatment process normally begins with detoxification and easing withdrawal symptoms is crucial when initiating treatment (NIDA InfoFacts, 2009). Thereafter, various pharmacological and behavioural treatments are introduced. Anti-depressants and anti-psychotics (Arias & Kranzler, 2008) are used to correct neurochemical imbalances in the brain (as discussed above). Interventions, to minimize the rewarding aspect of the drug have also been proposed i.e. pharmacological treatments that inhibit the drug's reinforcing effects as well as treatments that render the effects unpleasant (Maxwell, 2005). Cognitive-behavioural therapy, multidimensional family therapy and motivational interviewing and incentives are also implemented. Treatments within the criminal justice system are required in some cases. These treatments often succeed in preventing the offender from returning to criminal behaviour (NIDA InfoFacts, 2009). Ultimately relapse prevention is then implemented. Support systems and counselling are included in this process to assist the patient in maintaining a drug-free lifestyle while functioning as a productive member of society.

Although, the mechanisms by which METH exerts its effects have been identified and provides some insight to pharmacological treatment as well as pharmacological targets for treating METH, no recognised pharmacological treatment exists (Karila *et al.,* 2009). Though "prevention is better than cure", drug awareness campaigns often fail in preventing drug addiction, thus establishing an effective treatment plan for such addiction is crucial.

2.5.8. METH overdose

METH overdose can occur either intentionally or unintentionally and presents with a range of symptoms i.e. extreme agitation, rapid heart rate and respiration and hyperthermia

(Cruickshank & Dyer, 2009). METH overdose can also cause nausea and vomiting accompanied with seizures (Darke *et al*., 2008). The cause of death following direct METH abuse is generally due to multi-organ system failure i.e. cardiac, renal or hepatic failure (Vearrier *et al*., 2012; Cruickshank & Dyer, 2009). The neurological complications of METH may cause intracranial haemorrhage and status epilepticus which usually results in death. Thus, METH can cause death either directly, through neurological and/or cardiac complications or indirectly through suicidal events. METH overdose can also be acute or chronic. Acutely, an abuser may intentionally or unintentionally administer an extremely high dose of the drug, which results in life-threatening side-effects. A chronic METH overdose refers to the effects that are observed in long term abusers that use the drug on a regular basis.

2.6. Chemical and physical properties of METH and related pharmacokinetics

The chemical name for METH is *n-methyl-1-phenyl-propan-2-amine.* It is classified as a phenylethylamine psychostimulant, and includes psychoactive compounds such as amphetamine, ephedrine, pseudoephedrine and methylenedioxymethamphetamine (MDMA), depicted in Figure 2-3. Amphetamine is a contraction of α-methylphenethylamine which is an older description of this prototypical compound of which METH is the N-methyl derivative. The chemical structure of METH is also very similar to cocaine and mephedrone. The neurotoxicity of METH is also increased by mephedrone, although mephedrone does not damage DA nerve endings (Angoa-Pérez *et al.,* 2014).

Although most psychostimulants produce quantitatively similar effects, the duration of action of these effects may differ from one drug to another. Despite its structural similarities with other phenylethylamines, METH has a higher ratio of central to peripheral actions, as well as a faster onset of action than amphetamine and cocaine (Katzung *et al.,* 2009; Thrash *et al.,* 2009). The latter relates to its rapid distribution across the BBB, instant euphoria and long-lasting effects, due to its N-methyl group which makes the drug lipid soluble, thus improving its permeation over the BBB and increasing its resistance to degradation by MAO (Thrash *et al.,* 2009). Pharmacokinetically it is similar to ephedrine (Figure 2-3), while its metabolite, amphetamine enters the CNS even more readily (Katzung *et al.,* 2009). METH is metabolised mainly in the liver through the following three processes: (i) N-demethylation that produces amphetamine; this process is catalysed by cytochrome P450 2D6 and (ii) aromatic hydroxylation via cytochrome P450 2D6; this process primarily produces 4-hydroxymethamphetamine, as well as (iii) β-hydroxylation that produces norephedrine (Cruickshank & Dyer, 2009). After the drug has been metabolised by the liver it is excreted via the kidneys (Cruickshank & Dyer, 2009).

Amphetamine is a racemic mixture of phenylisopropylamine. METH exits as two enantiomers, namely dextrorotary and levorotary stereoisomers (mixture of *d*-and *l*-stereoisomers), where the dextrorotary isomer is considered the more biologically active optical isomer in the CNS, and the levorotary isomer has more peripheral activity (Cruickshank & Dyer, 2009). The *d*-stereoisomer is also up to 10 times more effective in affecting DAT function i.e. inhibiting DA reuptake. The structure of METH is analogous to amphetamine and mephedrone, both of which can be manufactured using the ephedrine or pseudoephedrine reduction method (Thrash *et al.,* 2009). The chemical structure of METH and derivatives are displayed in Figure 2-3.

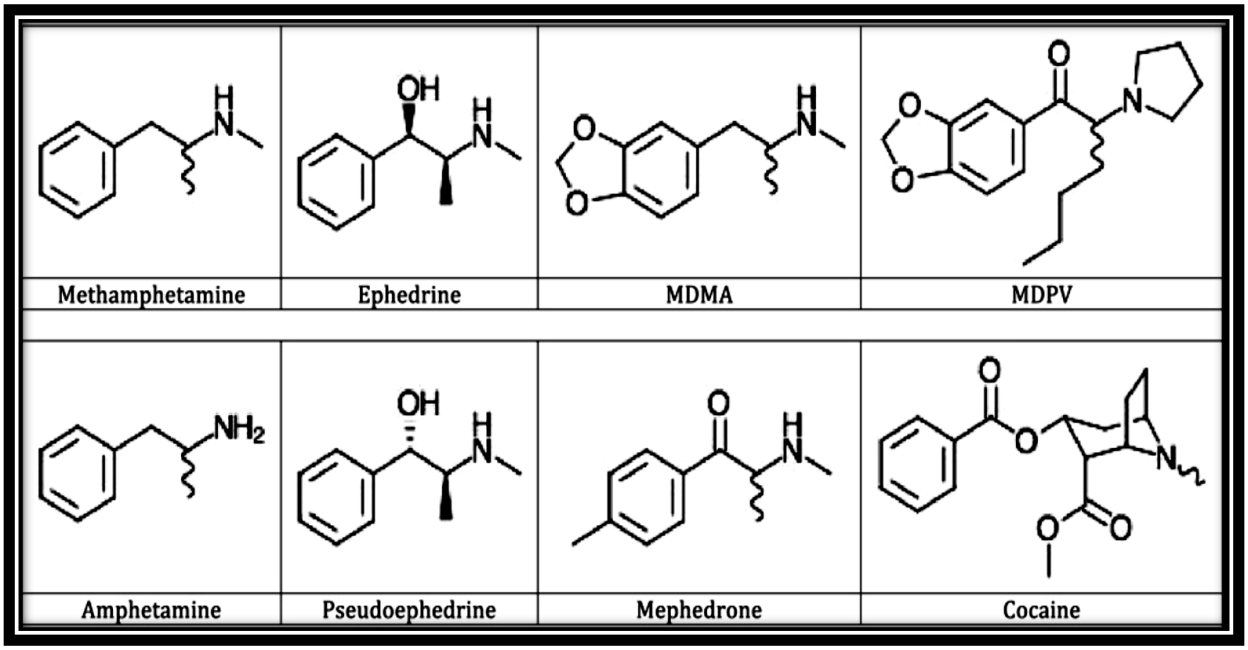

Figure 2-3: The chemical structure of METH and derivatives (Vearrier *et al.,* 2012).

METH is commonly found as a crystalline hydrochloride salt i.e. METH-HCl, which is depicted in Figure 2-4. METH-HCL is a more stable form and is identified as a clear colourless liquid at room temperature. METH-HCL crystals is freely soluble in water, alcohol and in chloroform.

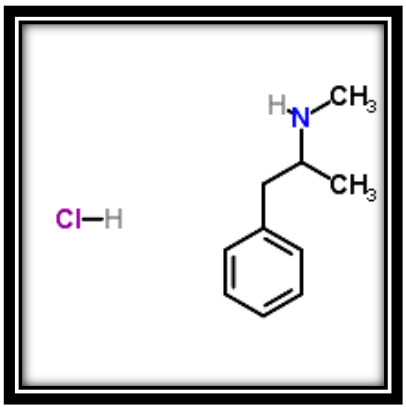

Figure 2-4: The chemical structure of methamphetamine hydrochloride (METH-HCL). Empirical formula: $C_{10}H_{15}N \cdot HCl$

METH-HCL possesses sympathomimetic properties with more potent central effects than amphetamine. It is transported into terminals via the monoamine transporters and causes the release of DA, NE, epinephrine and 5-HT. The release of DA produces the addictive properties of METH, while NE and epinephrine release are responsible for the cardiovascular adverse effects observed.

2.7. The pharmacodynamics of METH

2.7.1. METH and its effects on neurochemical markers (monoamines)

METH is a multi-effective drug and has been shown to increase the activity of catecholaminergic neurotransmission. Thus, it is a powerful releaser and indirect agonist of monoamines in the CNS, the result of which is an increase in cytoplasmic concentrations of DA, 5-HT and NA as well as histamine and adrenaline (Karila *et al.,* 2009). METH exerts these effects by blocking the intracellular vesicular monoamines transporter 2 (VMAT2); by decreasing expression of the DAT on the surface of the cell (Saha *et al*., 2014); by inhibiting the activity of MAO, and by increasing expression of tyrosine hydroxylase (Karila *et al.,* 2009). The specific mechanism of METH-induced DA release involves the distribution of neurotransmitters from synaptic vesicles via the vesicular monoamine transporter (VMAT2) to the neuronal cytoplasm and the reverse transport of dopamine through the plasma membrane transporter into the extracellular space as can be seen in Figure 2-5. Although the human body responds quickly to adapt to activation of these sympathetic pathways, sustained overstimulation of the stress response, caused by chronic METH use, may exceed the capacity to maintain normal neurotransmission.

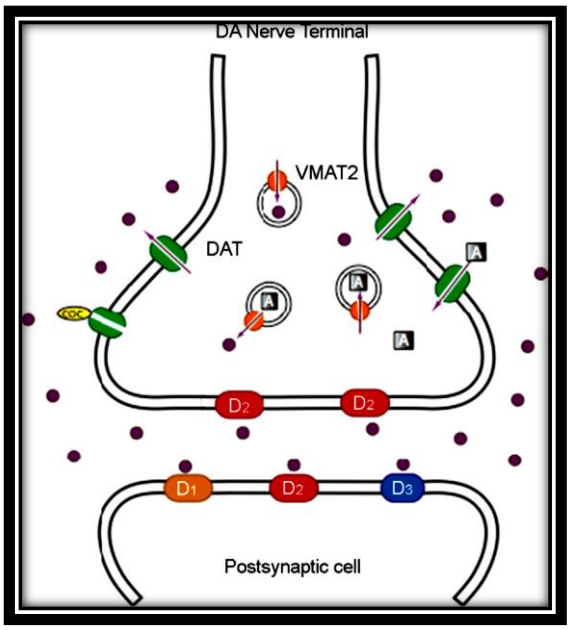

Figure 2-5: The dopaminergic synapse, including the pre- and post-synaptic terminals. Dopamine is packaged into vesicles in the presynaptic neuron via VMAT2. Dopamine in the synapse can bind to postsynaptic dopamine receptors, including the D_1, D_2, and D_3 receptors. D_2 receptors are also found at the presynaptic terminal, acting as a feedback mechanism to regulate dopamine release. METH acts at the DAT to alter normal DA receptor functions blocking the DAT, inhibiting uptake of dopamine into the presynaptic nerve terminal, thereby prolonging its effects in the synapse. In addition METH causes the release of DA from intracellular vesicles. A = Amphetamine; COC = cocaine; DAT = dopamine transporter; VMAT2 = vesicular monoamine transporter 2.
(Howell and Kimmel, 2008).

Thus, chronic and/or recurrent METH abuse may result in temporary or permanent disruptions in the dopaminergic system of the brain. Dopamine (DA) controls locomotion, motivation, cognition, and reward-associated functions in the brain (Saha *et al.,* 2014). Activation of the reward system is associated with an increased activity of DA-containing but not noradrenergic pathways. The long-term usage of METH also results in down-regulation of D_2 receptors and a decrease in the number of reuptake sites (Karila *et al.,* 2009). Thus, hypo-dopaminergic activity is typically seen. Hypo-activity of DA cells is expressed in the form of reduced extracellular DA levels and explains the neural basis of the dysphoric state observed in METH abusing individuals during the withdrawal phase (Melis *et al.,* 2005). Remarkably, this hypo-dopaminergic state outlasts the physical signs of withdrawal. It has been suggested that short-term treatment with D_2 receptor agonists restores the hypo-dopaminergic neuronal function and could be considered as a potential treatment for intermediate withdrawal phases (Melis *et al.,* 2005). These disturbances in DA activity may also predispose abusers to Parkinsonism (Tocharus *et al.,* 2010).

Due to structural similarity, METH is a substitute for monoamines at membrane-bound transporters. These include the DAT, norepinephrine transporter (NET) and serotonin transporter (SERT). The active forms of these transporters are cell surface integral membrane proteins (Cruickshank & Dyer, 2009). METH causes the redistribution of monoamines from storage vesicles into the cytosol by disrupting the pH gradient that otherwise drives accumulation of monoamine in these storage vesicles (Cruickshank & Dyer, 2009). Thus, the endogenous function of DAT, NET and SERT is then reversed, which results in the release of DA, NE and 5-HT from the cytosol into synapses. Synaptic monoamines are then available to stimulate postsynaptic monoamine receptors. METH also attenuates monoamine metabolism by inhibiting MAO (Cruickshank & Dyer, 2009).

2.8. Illicit METH retail markets, production of METH and related problems

As mentioned early in this chapter, METH is normally produced in primitive and clandestine laboratories. These laboratories can be found in kitchens, garages and even bedrooms (McKellar, 2005). METH is produced from inexpensive, easily accessible materials, including ephedrine or pseudoephedrine found in many over-the-counter (OTC) medicines, (Herman-Stahl *et al.,* 2007). The ephedrine or pseudoephedrine reduction method is commonly used to manufacture METH. For this reason access to pseudoephedrine has been restricted in the United Kingdom (UK) as well as in South Africa (SA). METH has been classified as a schedule 7 drug according to the Medicines and Related Substances Act 101 of 1965. The chemical similarities between METH, amphetamine, ephedrine and pseudoephedrine are depicted in Figure 2-3.

METH is sold by dealers or sellers to buyers or users. In addition to the direct effects of METH on abusers, indirect impacts are also of concern. The indirect effects of METH are related to the manufacturing process. A pseudoephedrine derived product is the main chemical ingredient or precursor that is required in the manufacturing of METH. Other ingredients like acetone, red phosphorous, ethyl alcohol and lithium metal are used and are flammable. Corrosive products like sulphuric acid, battery acid and sodium hydroxide can also be added to the ingredients (McKellar, 2005). Recipes for the manufacturing of METH are freely available on the internet. METH can be produced within 6 to 8 hours using hardware that can be easily dismantled and then hidden. Explosions and fires often break out resulting in casualties. Fires in METH labs also lead to the uncovering of these laboratories by law enforcement (Dipiro *et al.,* 2011). It has been reported that METH produced today is up to 6 times more effective than the METH produced in the 1960's (McKellar, 2005).

The general health and safety of communities are directly related to their involvement with drug-markets. METH also impact families, entire communities and local governments. Children living in homes where METH is produced or stored are constantly exposed to safety and health hazards. Physical, emotional, sexual abuse and neglect are commonly seen in these families. It has also been reported that children accidentally ingest some of the ingredients used for METH production and due to a higher respiration rate in children inhalation of toxic fumes render them more vulnerable to related respiratory difficulties and disease (McKellar, 2005).

2.9. METH as a prescription drug

METH is a registered prescription drug in the USA and can be used to treat a number of medical conditions including attention deficit hyperactivity disorder (ADHD), narcolepsy as well as short-term treatment for obesity. METH is however a controlled substance and can thus only be dispensed with a prescription. A weaker form of METH is sold as a prescription brand name *Desoxyn*® (methamphetamine hydrochloride) for weight control. Adderall® containing amphetamine is used to treat ADHD (Stedham *et al.,* 2007). Other psychostimulants used as therapeutics in the USA are depicted in Table 2-1. There are of course a number of concerns regarding the medical use of METH, including an increase in blood pressure, raised heart rate and respiration and an increase in core body temperature even at therapeutic doses (Stedham *et al.,* 2007). Particular also, due to the highly addictive properties of METH, Desoxyn®, Adderall® and other amphetamine of methamphetamine containing products are very rarely prescribed. It is therefore only used in extremely rare circumstances where no other treatment has shown efficacy.

Table 2-1: Psychostimulants used as a therapeutic intervention in the United States (Adapted from Howell & Kimmel, 2008)

Drug	Trade Names	Medical Indication
Amphetamine	Adderall® Dexedrine® Dextrostat®	ADHD, narcolepsy, weight control
Methamphetamine	Adipex® Desoxyn® Methedrine®	ADHD, weight control

2.10. The Flinders Sensitive Line (FSL) rat as a valid animal model of major depressive disorder (MDD)

Animal models of depression are used to study biological parameters and treatments that have been associated with major depression in humans. The purpose of developing an animal model is to study a disease or disorder where the main objective of an animal model is to replicate specific aspects of the human disorder under controlled experimental conditions.

The current study employed Flinder's Sensitive Line (FSL) and Flinder's Resistant Line (FRL) rats. FSL rats represent a well described and validated genetic animal model of depression (Overstreet & Wegener, 2013). The FSL rat strain is a valuable model as it mimics numerous biological and behavioural characteristics associated with depression (see Table 2-2) as well as increased stress responsiveness, as opposed to their inbred controls, the FRL rats (Overstreet & Wegener, 2013). The FSL rat was established at Flinders University, Australia, by genetically breeding Spraque-Dawley rats to exhibit super-sensitivity to cholinergic agents. Originally the FSL rats were developed to produce a line of rats that is genetically resistant to the effects of diisopropyl fluorophosphate (DFP) (organophosphate anticholinesterase agent). The breeding program for this line of rats then, un-expectantly, produced a strain of rats that were genetically more sensitive to DFP and, thus, named the Flinders Sensitive Line. Consequently the FRL line of rats was only more resistant to DFP relative of the FSL rats and not to their outbred control (Overstreet *et al.,* 2005).

Animal models can be evaluated on the basis of fulfilling three criteria: (1) face validity (strong phenomenological similarities and symptomatic appearance to the human illness), (2) construct validity (consistency with theoretical rationale suggested to underlie the human illness) and (3) predictive validity (similar responses to common treatments used in the human illness, or no response and no efficacy in humans) (Yadid *et al.,* 2000). It is well documented that the FSL rat resembles many characteristics of a depressed individual, including an increased sensitivity to cholinergic agonists (Overstreet *et al.,* 2005). Other similarities also exist (see Table 2-2) which makes this model extremely useful for translational research purposes.

Table 2-2: The similarities between the FSL rat strain and humans suffering from depression (Yadid *et al.,* 2000).

Characteristics	FSL rat strain	Depressed individuals
General activity	Reduced	Reduced
REM-sleep amount	Increased	Increased
REM-sleep latency	Reduced	Reduced
Appetite	Reduced	Reduced
Cognitive impairment	Yes/No?	Yes
Anhedonia	Yes	Yes
AD therapeutic response	Yes	Yes

The FSL rat displays reduced activity in a novel open field when compared to the Sprague-Dawley rats which relates to the reduced activity and psychomotor retardation seen in depressed patients (Table 2-2) (Overstreet *et al.,* 2005; Yadid *et al.,* 2000). FSL rats also exhibit elevated rapid eye movement (REM) sleep and a reduced latency between REM cycles as well as a decreased body weight and reduced appetite compared to the standard Sprague-Dawley strain. An increase in serotonergic activity results in reduced appetite and consequently the loss of weight that is often observed in depressed patients. Previous studies have reported that the FSL rat do not exhibit cognitive impairments under basal conditions which are commonly reported in depressed individuals (Overstreet *et al.,* 2005), however, recent studies indicate that possible cognitive impairments do exist in the FSL rat (Eriksson *et al.,* 2012; Husum *et al.,* 2001). Abildgaard (2011) and colleagues found that the depressive-like phenotype of FSL rat is associated with impairment in novel object recognition memory (Abildgaard *et al.,* 2011). In addition, study conducted in 2012 indicated that the FSL rat line can be employed in studies as a model of reversible impairments in emotional processing and memory. The FSL rat also displays predictive validity for evaluation of drugs supporting cognitive performance (Eriksson *et al.,* 2012). FSL rats and depressed patients both present with an inability to experience reward (anhedonia) (face validity) (Yadid *et al.,* 2000). Thus, the FSL rat model of depression has a substantial degree of face validity.

Neurochemical and pharmacological evidence have suggested that the FSL rat also exhibits neurochemical changes consistent with the cholinergic, serotonergic and dopaminergic models of depression (construct validity) (Overstreet *et al.,* 2005; Yadid *et al.,* 2000; Brand *et al.,* 2012) as well as deficits in glutamatergic signalling (Overstreet & Wegener, 2013). The variety of serotonergic abnormalities includes super-sensitive hypothermic responses to 5-

HT_{1A} agonists, and increased levels of 5-HT in the limbic regions of the brain (Yadid *et al.,* 2000). Researchers have also confirmed a working hypothesis that cholinergic super-sensitivity provides the foundation for a neurochemical association between depressive disorders and asthma and irritable bowel (Overstreet *et al.,* 2005). The FSL rat also display significantly decreased levels of neurotrophic factor BDNF and vascular endothelial growth factor (VEGF) and congruently a reduction in neuronal and synapse numbers (Overstreet & Wegener, 2013). Recent data have also indicated the presence of single-nucleotide polymorphisms (SNP's) in these animals that are consistent with MDD (Melas *et al.,* 2013). SNP's in genes vital to T-cell function were found to be associated with MDD (Hashmi *et al.,* 2013). Neuropeptide Y (NPY) is one of the few candidate genes that indicates reproducibility among genetic association studies of depression and it is hypothesised that hippocampus dysregulation of NPY in the FSL rat may be caused by these functional SNP's in the promoter region, similar to what is seen in humans (Melas *et al.,* 2013). The FSL rat also displays an increase in immobility in the forced swim test (FST) that can be reversed following chronic (14 days) treatment with an antidepressant drug (predictive validity) (Table 2-2). Thus, a substantial amount of data supports the face, predictive and constructive validity of the model (Yadid *et al.,* 2000).

2.11. Executive summary

METH abuse is a fast growing and serious global problem that has been classified as an epidemic (Cruickshank & Dyer, 2009). This epidemic is accompanied by significant public health implications and economic costs (Stomberg & Sharma, 2012). Attributing to this epidemic is the fact that METH is simple to synthesize from inexpensive and readily available materials, including over the counter drugs. Consequently vulnerable individuals have easy access to METH, so that its abuse has increased globally. In fact, METH is the second most popular illicit drug world-wide (Cruickshank & Dyer, 2009) with an estimated 35 million people regularly abusing the drug (Vos *et al.,* 2010).

METH produces a unique pseudodepressive state due to its ability to directly affect monoamine regulation. Continued use may also result in severe weight loss, dermatological decay, uncontrollable rage, paranoia and cognitive impairments. Individuals abusing METH experience negative social and emotional consequences, including the loss of family relationships, the inability to participate in educational and work activities and involvement in criminal activities. However, as the drug compromises decision-making with an increase in impulsive risk-taking behaviour, these severe consequences do not seem to invoke cessation of the drug (Gowin *et al.,* 2013).

It is commonly reported that substance abusers are mostly among young adults, especially between the ages of 18 to 25 years (Cohen, 2014). METH abuse during this developmental stage can increase the probability of adverse effects on neurodevelopment and may produce early-life changes that can result in neurocognitive changes later in life. Indeed, depression is associated with early-life adversity (Modgil *et al.,* 2014), and since METH abusers are often individuals that have come from a disadvantaged background, they represent a population already at high risk for developing a mood disorder due to the combined impact of METH plus early-life adversity, a paradigm known as the double hit hypothesis (Nabeshima & Kim, 2013). Thus, the current study will investigate the effects of chronic METH administration during pre-adolescence in genetically predisposed individuals (FSL rats) versus a control population (FRL rats) and the subsequent development of depression related bio-behavioural changes later in life. Since METH-induced depression is one of the key symptoms that require treatment during the withdrawal phase as well as later on in life, this study will focus on the development of depressive-like behaviour following chronic (16 days) administration of METH or vehicle during pre-adolescence immediately and 25 days post withdrawal. These behavioural results will be discussed in article format in Chapter 3. In addition, the study will investigate regional brain biomarkers related to the neurobiological basis of depression, viz. the levels of DA, NE and 5-HT and their metabolites in the frontal

cortex. The results of the latter analyses will be presented as additional and supplementary data in Addendum B. Specific behavioural tests will be used to evaluate cognitive functioning (memory), locomotor activity, anxiety-like behaviour and depressive-like behaviour in the animals.

Chapter 3 - Scientific Article

This dissertation is presented in article format, as recognised by the North-West University. This implies that the bulk of results are presented as a manuscript for submission to an appropriate peer review journal. The current chapter therefore presents the most significant data from the study as a research article. The article is titled:

"The long-term effects of methamphetamine exposure during pre-adolescence on depressive-like behaviour in stress-sensitive rats"

and has been prepared for submission to the **International Journal of Developmental Neuroscience** as a full-length research report. This chapter has been prepared according to the instructions to the author of this particular journal (see Addendum C). Instruction to the author can also be found at the following website:

http://www.elsevier.com/wps/find/journaldescription.cws_home/712?generatepdf=true

The references used in preparing the article are thus listed at the end of the report and not at the end of the dissertation and also according to the bibliographic style prescribed by the journal and not as prescribed by the NWU for dissertations.

The article will begin with the title page that includes the title of the article, contributing authors' names and affiliations and present addresses and the corresponding author, followed by the Abstract on a separate page. The article is then further subdivided into the following sections, the Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgements and References, Legends to Tables, Tables, Legends to Figures and Figures. However, to ease reading, all figures and tables have been included in the text and not at the end of the article as required by the Journal.

M. Mouton assumed all the behavioural, neurochemical and statistical analyses and composed the first draught of the manuscript. Prof C.B Brink designed and supervised the study and assisted in the interpretation of the study data, as well as finalized the manuscript for publication. Prof B.H Harvey provided guidance to the study design and assisted in the finalization of the article.

Title of article

The long-term effects of methamphetamine exposure during pre-adolescence on depressive-like behaviour in stress-sensitive rats

Names of authors

Moné Mouton[1], Brian H. Harvey[2], Christiaan B. Brink[1].

Affiliation of authors

[1]Division of Pharmacology, School of Pharmacy, Faculty of Health Sciences, North-West University, Potchefstroom, 2520, South Africa and [2] Centre of Excellence for Pharmaceutical Sciences, School of Pharmacy, Faculty of Health Sciences, North-West University, Potchefstroom, 2520, South Africa.

Corresponding author

Christiaan B. Brink, PhD

Internal box 16, Unit for Drug Research and Development, School of Pharmacy, North-West University (PUK), Potchefstroom, 2520, South Africa. Tel:.018 2992234, Fax: 0182992225, Email: Tiaan.Brink@nwu.ac.za

Author names, affiliations and addresses

Moné Mouton

[1]Division of Pharmacology, School of Pharmacy, Faculty of Health Sciences, North-West University, Potchefstroom, 2520, South Africa.

Christiaan Beyers Brink

[1]Division of Pharmacology, School of Pharmacy, Faculty of Health Sciences, North-West University, Potchefstroom, 2520, South Africa.

Brian Herbert Harvey

[2]Centre of Excellence for Pharmaceutical Sciences, School of Pharmacy, Faculty of Health Sciences, North-West University, Potchefstroom, 2520, South Africa.

Abstract

Methamphetamine (METH) is a derivative of amphetamine and a synthetic psychostimulant that is mostly abused for recreational purposes. Said abuse has developed into an epidemic in many countries including South Africa. Early-life exposure to METH and late-life development of depression and the mechanisms involved requires further investigation.The current study investigated the effects of chronic METH exposure during pre-adolescence in genetically predisposed individuals (Flinders Sensitive Line or FSL rats) versus a control population (Flinders Resistant Line or FRL rats). The subsequent development of depression related bio-behavioural changes were then studied later in life. Since METH-induced depression is one of the key symptoms that require treatment during METH withdrawal as well as later on in life, this study focused on behavioural changes following chronic (16 days) administration of METH or vehicle between post-natal day (PnD) 19 and 34 immediately and 25 days post treatment (after the withdrawal phase). The animals were tested for depressive-like behaviours as well as deficits in locomotor activity, social interactive behaviour and recognition memory.

METH induced antidepressant-like behaviour in both FSL and FRL rats in the forced swim test (FST), as determined immediately before withdrawal. However, METH induced significant depressogenic effects when assessed 25 days post-treatment in METH-treated FRL and FSL rats. METH reduced locomotor activity on PnD35 in both FSL and FRL rats, although METH did not display any significant differences after wash-out. Furthermore, METH significantly lowered social interaction behaviour (staying together) in both FRL and FSL rats, both immediately following drug treatment (PnD35) and after withdrawal (PnD60), while it significantly deceased self-grooming time on PnD35 only. METH treatment enhanced exploration of the familiar object in the novel object recognition test (nORT) in FSL and FRL rats before withdrawal on PnD35 as well as after withdrawal on PnD60. Thus, early-life exposure to METH may result in acute antidepressant-like effects immediately after chronic exposure, however it is depressogenic when assessed following an extended withdrawal period. We did observe a predisposition in individuals with a depressive-like phenotype (i.e. in FSL vs. FRL rats) where METH-treated FSL rats displayed a trend towards greater immobility than METH-treated FRL rats on PnD60, suggesting that individuals that are genetically predisposed to depression are at a greater risk to developing depression after METH exposure relative to individuals that do not present with this predisposition.

Keywords

Methamphetamine, Finders Sensitive Line, Flinders Resistant Line, Depression, Early-life exposure.

3.1 Introduction

Methamphetamine (METH) is a derivative of amphetamine and a synthetic psychostimulant that is mostly abused for recreational purposes and presents with highly addictive properties. METH abuse is a fast growing global problem that has been classified as an epidemic (Cruickshank & Dyer, 2009; Yamamoto *et al.,* 2010). Contributing to this epidemic is the fact that METH is easily synthesized at low cost using house-hold chemicals and over the counter drugs. METH has been considered the second most popular illicit drug world-wide (Cruickshank & Dyer, 2009) and it is estimated that more than 35 million people are regularly abusing METH (Vos *et al.,* 2010; Mehrjerdi *et al*., 2014).

METH produces a unique pseudodepressive state due to its ability to directly affect monoamine regulation. The symptoms of this state includes anhedonia, fatigue, sleep abnormalities, loss of appetite, lack of motivation, irritability and poor concentration, and thus relate to many of the features of major depressive disorder (MDD) (McKetin et al., 2011). Early-life exposure to adversity, such as drug abuse, has been shown to aid the development of late-life behavioural abnormalities and neurodegenerative diseases (Strauss *et al.,* 2014; Modgil *et al.,* 2014). Studies suggest a strong relationship between exposure to adverse environmental factors early in life and the later development of a neuropsychiatric disorder, such as depression (Modgil *et al.,* 2014). Extensive research has provided evidence that continued and repeated exposure to psychostimulants, such as METH, results in sensitisation of the reward- and psychomotor effects of the drugs (Achat-Mendes *et al.,* 2003). This is thought to be as a result of neurobiological changes, particularly in the mesolimbic dopamine (DA) system. Drug-induced changes in these reward circuits are long-lasting, and include alterations in gene transcription, RNA and protein synthesis and modifications of neuron morphology (Achat-Mendes *et al.,* 2003).

It has been documented that early-life (pre-adolescent and adolescent) exposure to psychostimulants impairs neural plasticity and is associated with a decrease in the rewarding effect of drug abuse (Achat-Mendes *et al.,* 2003). Prenatal exposure to METH in children results in deficits in sustained attention, long-term spatial and verbal memory as well as visual motor integration during neurocognitive testing (Thompson *et al.,* 2009). It has been documented that parental depression may have negative effects on child development and it is crucial that parental depression and/or anxiety be recognised as a child's earliest adverse life event (Koehler, 2006). Maternal stress affects numerous aspects of cognitive and psychosocial function in the foetus that usually persists beyond infancy. Emotional and behavioural deficits have been observed in preschool children that have been subjected to maternal stress. An array of evidence thus demonstrates that early life adversity contributes

significantly to the susceptibility of adulthood psychopathology, medical illnesses (such as depression), and maladaptive psychosocial functioning (Koehler, 2006) as well as altered hippocampal plasticity (Ansorge *et al.,* 2007).

METH also impairs novel location and novel object recognition memory when administered to rats during phases associated with hippocampal development (Siegel *et al.,* 2011). In addition, neuroimaging studies in humans has demonstrated smaller striatum and hippocampus volumes, a decrease in dopamine D_2 receptor number and a lower dopamine transporter density (Thompson *et al.,* 2009), similar to what is seen in depressed individuals. Although numerous pre-clinical studies have focused on prenatal exposure to METH and its late-life effects, few studies have focused on the consequences of early-life (pre-adolescent and adolescent) exposure to METH. Since it has been commonly reported that substance abusers are mostly among young adults, especially between the age of 18 to 25 years (Cohen, 2014), it is crucial that the adverse effects during this developmental stage are further investigated.

Since METH-induced depression is one of the key symptoms that require treatment during the withdrawal phase as well as later on in life, this study will focus on the development of depressive-like behaviour (in an animal model) following chronic (16 days) METH versus vehicle administration during pre-adolescence. Since depression is associated with early life adversity (Modgil *et al.,* 2014), and that METH abusers are often individuals that have come from a disadvantaged background, they represent a population already at high risk for developing a mood disorder due to the combined impact of METH plus early life adversity, a paradigm known as the double hit hypothesis (Nabeshima & Kim, 2013). For this reason, we will use the Flinders Sensitive line rat (FSL), a genetic rodent model of depression, to assess whether genetic predisposition plus METH exposure presents with a greater risk for developing depressive like behaviour later in life. The effect of METH was assessed using a battery of behavioural tests that were then scored and analysed.

The working hypothesis of this study is that chronic treatment with METH will induce depressive-like behaviours. Moreover, we will also consider whether combined METH plus genetic predisposition in individuals with a depressive-like phenotype poses a greater risk for developing late-life behavioural anomalies akin to MDD. We expect that this study will improve our understanding of the neurobehavioural dysfunction brought about by early-life exposure to METH and the mechanism of how METH abuse may precipitate or worsen depressive-like symptoms. It will also produce a working hypothesis for treatment options and direct further investigations and studies.

3.2 Materials and Methods

3.2.1 Animals

Male and female Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats were supplied by the Vivarium at the Pre-Clinical Drug Development Platform (PCDDP) of the North-West University. Animals were housed and bred at the Vivarium of North-West University (NWU), with the original colony obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA.

The animals were housed 4 rats per cage under conditions of constant temperature (22 ± 1°C) and humidity (50 ± 10%) with a 12:12-h light/dark cycle (lights on at 06:00 and lights off at 18:00). Food and water were provided *ad libitum*. Cages were cleaned and bedding (sawdust) replaced weekly.

All experiments conformed to the guidelines of the National Institutes of Health for the care and use of laboratory animals, and were approved by the Animcare Animal Research Ethics Committee of the North-West University (ethics approval no. NWU-000105-11-S5 – Approved: 07/10/2011). All animals were maintained according to a code of ethics in research, training and testing of drugs in South Africa and complied with national legislation.

3.2.2 Drug treatment

Methamphetamine hydrochloride (METH HCl) was purchased from Sigma Aldrich, Missouri, USA, imported under South African permit no. IMP/626/2011, kept under licence no. POS107/2011/2012 and usage logged as prescribed by national legislation. METH HCl crystals were dissolved in saline (vehicle) for subcutaneous administration. FSL and FRL rats were treated with vehicle or METH HCl twice daily at 09:00 and again at 15:00 from postnatal day nineteen (PnD19) to postnatal day thirty-four (PnD34) (pre-puberty, with sexual maturity reached on PnD35). METH HCl was administered in an escalating dose regimen during the 16 day period (mimicking binging behaviour in humans), with every dose escalating in increments of 0.2 mg/kg from 0.2 to 6.0 mg/kg (i.e. 0.2, 0.4, 0.6, ... , 5.6, 5.8, 6.0, 6.0, 6.0 mg/kg). Each animal was weighed before injection and the appropriate dose calculated. Saline-treated animals received a fixed dose of 0.2ml saline SC twice daily.

3.2.3 Behavioural Testing

After the 16-day period of drug administration, the study investigated behavioural effects of the treatments, implementing the novel object recognition test (nORT) first, thereafter the social interaction test (SIT), then the locomotor activity test and finally the forced swim test (FST). These were performed as a battery of tests either directly after the injection period on PnD35, or later in life after drug wash-out (withdrawal) in early adulthood on PnD60. All the behavioural tests were performed on each treatment group respectively. Since rats are nocturnal animals, behavioural testing commenced one hour after the start of the dark cycle i.e. 19:00.

3.2.3.1 Novel Object Recognition Test (nORT)

The novel object recognition test (nORT) is used to evaluate declarative memory in rodents. This test relies on the principle that rats prefer to explore a novel (new) object relative to a familiar one. The apparatus comprised of a box made of opaque, black Perspex, and with the floor dimensions 500 mm x 500 mm, and a 400 mm high surrounding wall. Two heavy objects, which cannot be moved by the rat, were placed inside the box.

The habituation trial commenced two days prior to the night of testing when the rats were given the opportunity to explore the empty nORT boxes (one rat per box) for 10 minutes. Thereafter, on the day of testing, the acquisition trial was conducted. The rats were given the opportunity to explore two identical, steady objects in the nORT box for a period of five minutes. These objects were identical in shape, size and colour and placed 10 cm from the box walls in two adjacent corners. The rats were then returned to their home cages for a 1.5 hour inter-trial interval (90 minutes). The nORT box and objects were cleaned with 10% ethanol to remove any olfactory cues. The animals were then subjected to the last trial, named the retention trial. The rat were returned to the test box where they were presented with two objects, one object identical to the object in the first exploration trial while the second object was novel. The new (novel) object has a different shape, size and colour and was therefore unfamiliar to the rat. Again the rats were allowed to explore the objects for five minutes, whereafter the rats was removed. Object exploration was defined as sniffing, licking or touching the objects with forepaws (Grayson *et al.,* 2007). Sitting near the object was not considered exploration. The exploration time (seconds) of each object in each trial was scored manually from the recorded videos using two stopwatches. With memory intact, rats tend to remember the familiar object and show a tendency to spend more time exploring the novel object. A decrease in the exploration time of the novel object is regarded as being indicative of impaired memory performance.

3.2.3.2 Social interaction test (SIT)

The SIT is used to measure explorative and social interactive behaviours between pairs of rats in an open field test (OFT) arena. The apparatus consists of a black square (100 cm x 100 cm x 40 cm) arena with opaque walls. The floor is divided into 25 cm x 25 cm equal squares (Sherif & Oreland, 1995) with the arena illuminated with red light (40 lux). A video camera placed above the arena records the activity of the animals for later analysis.

A pair of unfamiliar rats was placed in the arena together. The time spent in active social interactive behaviours, self-directed behaviours and explorative behaviours were then scored for a period of 10 minutes while the researcher remained outside the testing room during the testing period to avoid distracting the animals. The arena was subsequently cleaned with 10 % ethanol solution between tests to eliminate olfactory cues (Ferdman *et al.,* 2007). Both social interactive behaviour ("staying together") and self-directed behaviour ("self-grooming") were scored. Staying together is scored as one entity for both rats whereas self-grooming is scored for each individual rat. Less time spent in social interaction and increased self-grooming is considered indicative of raised anxiety levels.

3.2.3.3 DigiScan (Locomotor Activity)

The DigiScan® is an instrument used to measure spontaneous locomotor activity. The Digiscan (Accuscan Instruments Inc. Columbus, OH, USA) uses various infrared sensors placed strategically around the box to detect and measure activity within the cage (Cao *et al.,* 2002). Each open field cage presents with 16 infrared light beams aligned in the horizontal X and Y axes. Infrared sensors within the box detect both the activity levels of the rats and their location within the cage where the activity is displayed. The rat is placed in the centre of the chamber and consequently the horizontal activity, vertical activity (rearings) as well as the total distance travelled by the animal is measured for a period of five minutes. Activity is measured by recording the number of beam breaks caused by the animal. In particular, locomotor activity is used to distinguish between altered locomotor activity and altered psychomotor activity as a possible cause for any changes in immobility in the forced swim test (see below).

3.2.3.4 Forced Swim Test (FST)

The FST is used to assess depressive-like behaviour in rodents (Abildgaard *et al.,* 2011; Blokland *et al.,* 2012). The FST is regarded as a screening test for antidepressant activity or depressive-like behaviour, and is not a translational model of depression. Although the immobility behaviour of the animals is caused by the test itself and is a direct reaction to the test procedure, the immobility does not persist after the test has concluded or outside the

test situation. The FST does not cause an induction of a depressive state, however there are some components of construct validity i.e. stress-inducing conditions as well as a deceased behavioural output (Castagné *et al.,* 2009).

The rats were individually placed in a water-filled (25˚C ±2˚C) transparent plastic cylinder from which they cannot escape (height x 50 cm ; diameter x 25 cm ; water depth x 30 cm) and allowed to swim for a period of 7 minutes. Video monitoring was used to record the behaviour of the animals, with active scoring undertaken during the middle 5 minutes. The following activities were noted and scored (Blokland *et al.,* 2012): immobility (assuming an immobile position, floating, no movements other than to keep their nose or mouths above the water surface to breath), swimming (actively making swimming movements across the surface of the cylinder), and climbing (upward movements with the front paws in and out of the water surface, clawing against the side of the cylinder in an attempt to escape).

3.2.4 Statistical Analysis

When comparing only two data points, the Student's t test was used. However, for multiple comparison of data, the two-way ANOVA was used, and when this analysis indicated interaction between the main factors (i.e. rat line and drug treatment, respectively), it was followed by the Tukey posthoc. In all cases GraphPad Prism® version 6 (GraphPad Software, San Diego California USA, www.graphpad.com) was used for statistical analysis and graphical presentations. A 5% confidence limit for error was taken as statistically significant ($p<0.05$). The Statistical Consultation Service of the North-West University was consulted before finalization of analyses.

3.3 Results

3.3.1 The forced swim test (FST)

Figure 3-1 displays the immobility time for vehicle- and METH-treated FSL and FRL rats in the FST. Figure 3-1A displays measurements at PnD35 (i.e. 24 hours after the last dose) and Figure 3-1B at PnD60 (i.e. long-lasting effects after washout).

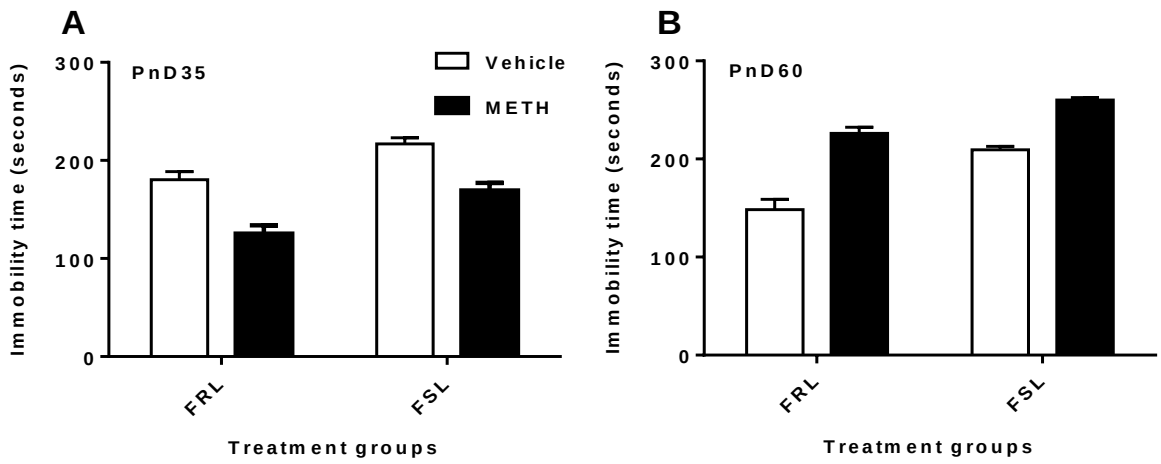

Figure 3-1: The effect of vehicle versus chronic methamphetamine (METH) exposure in Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats on immobility time (seconds) in the forced swim test. Immobility, as marker of depressive-like behaviour, was evaluated on PnD35 (A) and PnD60 (B). Data points represent the mean ± S.E.M.

In Figure 3-1A the two-way ANOVA of the data ($F(1, 49) = 0.2335$, $P = 0.6311$) indicates that there was no statistically significant interaction between line of rat and drug treatment regarding immobility, hence no post hoc analysis is allowed. Both main factor effects were statistically significant, i.e. the line of rats (FSL and FRL) ($F(1, 49) = 26.52$, $P < 0.0001$) and drug treatment (METH or VEH) ($F(1, 49) = 43.28$, $P < 0.0001$).

In Figure 3-1B the two-way ANOVA of the data ($F(1, 44) = 3.954$, $P = 0.0530$) indicates that there was no statistically significant interaction between line of rat and drug treatment regarding immobility, hence no post hoc analysis is allowed. Both main factor effects were statistically significant, i.e. the line of rats $F(1, 44) = 49.41$, $P < 0.0001$) and also drug treatment $F(1, 44) = 90.39$, $P < 0.0001$).

It can be seen in Figure 3-1 that the FSL rats displayed enhanced immobility relative to FRL rats in the FST on PnD35 and PnD60, both before and after METH withdrawal. Chronic METH exerts antidepressant-like effects (decreased immobility) prior to withdrawal on

PnD35 (adolescence) in both FRL and FSL rats. Following 25 days withdrawal on PnD60, METH exerts depressive-like effects (enhanced immobility) in FRL and FSL rats, thereby also further augmenting the enhanced depressive-like behaviour in genetically predisposing FSL rats.

3.3.2 Locomotor activity test

Figure 3-2 displays the total distance travelled by vehicle- and METH-treated FSL and FRL rats respectively, in the Digiscan® animal activity monitor (locomotor activity test). Figure 3-2A displays measurements at PnD35 (i.e. 24 hours after the last dose) and Figure 3-2B at PnD60 (i.e. long-lasting effects after washout).

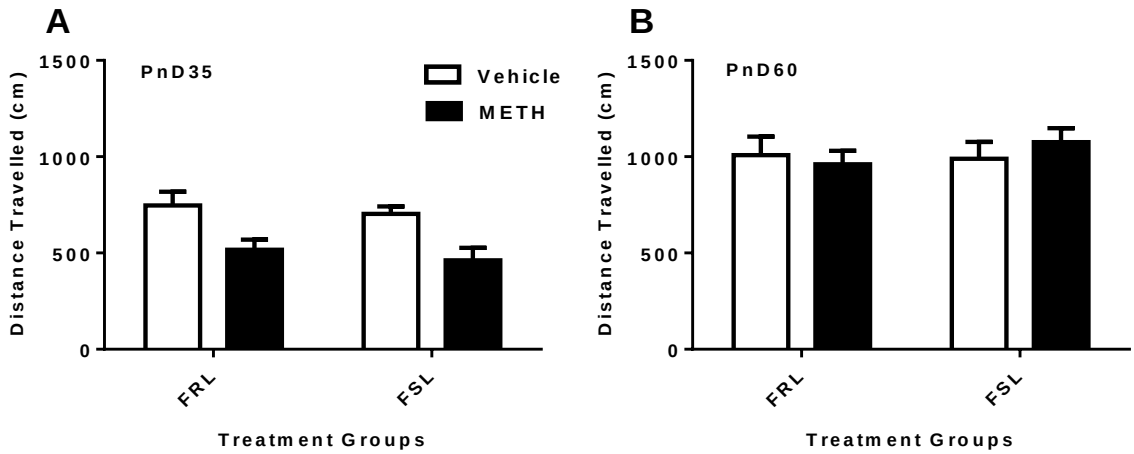

Figure 3-2: The effect of vehicle versus chronic methamphetamine (METH) exposure in Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats on locomotor activity in the Digiscan Apparatus. The total distance travelled was evaluated on PnD35 (A) and PnD60 (B). Data points represent the mean ± S.E.M.

In Figure 3-2A the two-way ANOVA of the data ($F(1, 52) = 0.01303$, $P = 0.9095$) indicates that there was no statistically significant interaction between the line of rats and drug treatment regarding locomotor activity, hence no post hoc analysis is allowed. The main factor effect of line of rat was also not significant $F(1, 52) = 0.7618$, $P = 0.3868$), whereas the drug treatment effect was statistically significant ($F(1, 52) = 17.49$, $P = 0.0001$) on PnD35.

In Figure 3-2B the two-way ANOVA of the data $F(1, 45) = 0.6124$, $P = 0.4380$) indicated that there was no statistically significant interaction between the line of rats and drug treatment regarding locomotor activity, hence no post hoc analysis is allowed. Both the main factor effects of line of rat ($F(1, 45) = 0.3190$, $P = 0.5750$) and drug treatment $F(1, 45) = 0.05259$, $P = 0.8197$) was not significant.

It can be seen in Figure 3-2 that METH reduced locomotor activity in both FSL and FRL rats on PnD35, but not on PnD60. The effect of METH on locomotor activity was similar in FSL and FRL rats.

3.3.3 The social interaction test (SIT)

Figure 3-3 displays the time spent in social interaction (staying together) and self-directed (self-grooming) behaviour by vehicle- and METH-treated FSL and FRL rats in the social interaction test (SIT). Figure 3-3 A and C displays measurements at PnD35 (i.e. 24 hours after the last dose) and Figure 3-3 B and D at PnD60 (i.e. long-lasting effects after washout).

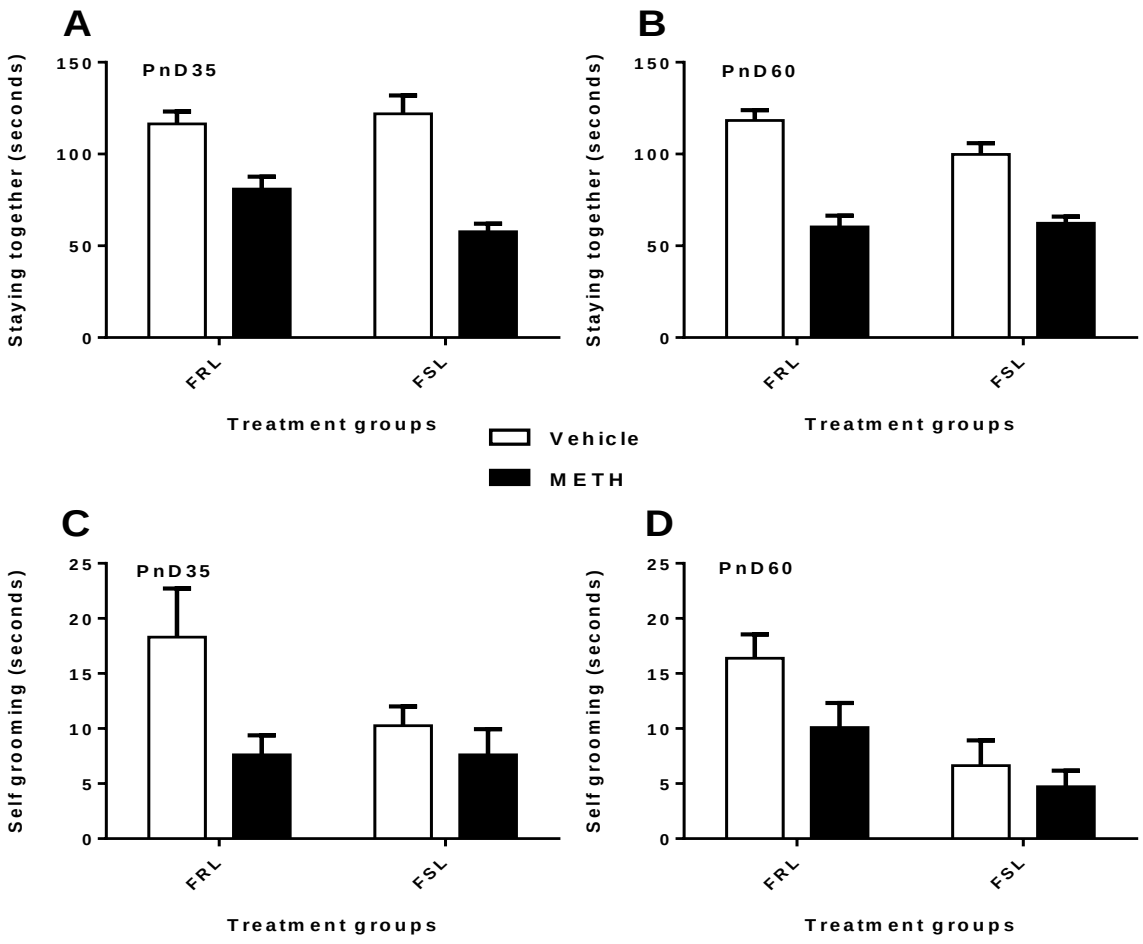

Figure 3-3: The effect of vehicle versus chronic methamphetamine (METH) exposure in Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats on social interaction and self-directed behaviour. Time spent in social interactive behaviours (staying together) (A and B) and time spent in self-directed (self-grooming) behaviour (C and D) was evaluated on PnD35 (A & C) and PnD60 (B & D). Data points represent the mean ± S.E.M.

In Figure 3-3A the two-way ANOVA of the data ($F(1, 28) = 3.568$, $P = 0.0693$) indicates that there was no statistically significant interaction between line of rat and drug treatment regarding staying together (social interaction behaviour) on PnD35, hence no post hoc

analysis is allowed. The main factor effects of line of rat was also not significant F (1, 28) = 1.350, P = 0.2552). However, METH treatment significantly lowered the amount of time that the rats stayed together (F (1, 28) = 42.89, P < 0.0001) on PnD35.

In Figure 3-3B the two-way ANOVA of the data (F (1, 25) = 3.448, P = 0.0751) indicated that there was no statistically significant interaction between line of rat and drug treatment regarding staying together (social interaction behaviour) on PnD60, hence no post hoc analysis is allowed. The main factor effect of line of rat was also not significant (F (1, 25) = 2.239, P = 0.1471), although METH treatment did statistically significantly decrease the amount of time that the rats stayed together F (1, 25) = 75.27, P < 0.0001) on PnD60.

In Figure 3-3C the two-way ANOVA of the data (F (1, 52) = 2.376, P = 0.1292) indicated that there was no statistically significant interaction between line of rat and drug treatment regarding self-grooming (self-directed behaviour) on PnD35, hence no post hoc analysis is allowed. The main factor effect of line of rat was also not significant (F (1, 52) = 2.359, P = 0.1307), however METH treatment did decease self-grooming time statistically significantly (F (1, 52) = 6.534, P = 0.0135) on PnD35.

In Figure 3-3D the two-way ANOVA of the data (F (1, 45) = 1.088, P = 0.3024) indicated that there was no statistically significant interaction between line of rat and drug treatment regarding self-grooming (self-directed behaviour) on PnD60, hence no post hoc analysis is allowed. The main factor effect of line of rat F (1, 45) = 12.94, P = 0.0008) was statistical significant, although the effect of drug treatment was not statistically significant F (1, 45) = 3.807, P = 0.0573).

It can be seen in Figure 3-3 that METH significantly lowered the amount of time that both FSL and FRL rats stayed together (social interactive behaviour) on PnD35 and PnD60. METH also decreased self-grooming time (self-directed behaviour) in both FSL and FRL rats on PnD35, but not on PnD60.

3.3.4 The novel object recognition test (nORT)

Figure 3-4 displays the percentage time spent by vehicle- and METH-treated FSL and FRL rats exploring the familiar and novel objects, respectively, in the novel object recognition test (nORT). Figure 3-4 A and B display measurements at PnD35 (i.e. 24 hours after the last dose) and Figure 3-4 C and D at PnD60 (i.e. long-lasting effects after washout).

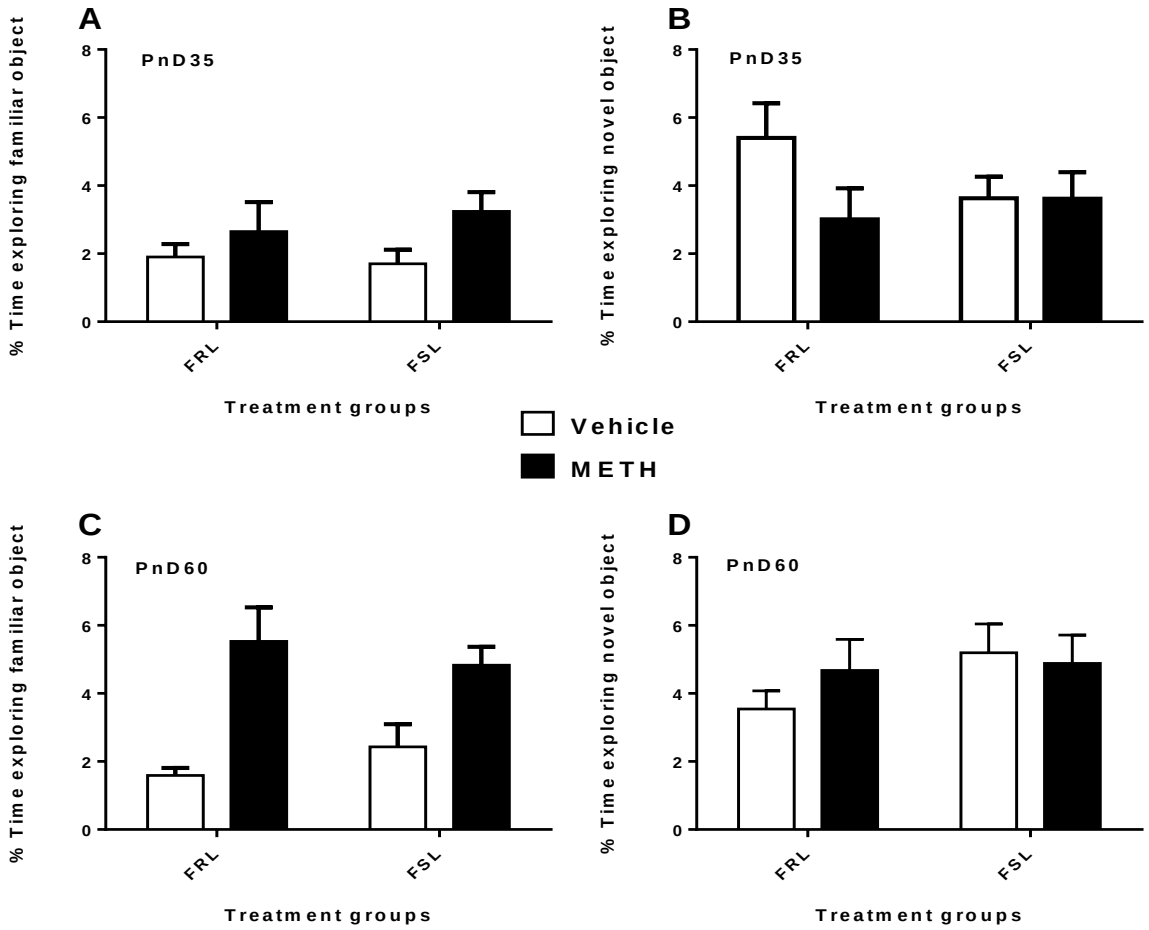

Figure 3-4: The effect of vehicle versus chronic methamphetamine (METH) exposure in Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats on % time spent exploring the familiar (A & C) and novel (B & D) objects, respectively, and also at (A & B) PnD35 and (C & D) PnD60, respectively, in the novel object recognition test. Data points represent the mean ± S.E.M.

In Figure 3-4A the two-way ANOVA of the data ($F(1, 49) = 0.5070$, $P = 0.4798$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding time sent exploring the familiar object on PnD35, hence no post hoc analysis is allowed. The main factor effects of line of rat was also not significant ($F(1, 49) = 0.1228$, $P =$

0.7275), however METH treatment significantly increased the time spent exploring the familiar object in both rat strains relative to vehicle controls (F (1, 49) = 4.167, P = 0.0466).

In Figure 3-4B the two-way ANOVA of the data (F (1, 49) = 2.079, P = 0.1557) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding time sent exploring the novel object on PnD35, hence no post hoc analysis is allowed. Both the main factor effects of line of rat (F (1, 49) = 0.4922, P = 0.4863) and drug treatment (F (1, 49) = 2.119, P = 0.1519) was not statistically significant.

In Figure 3-4C the two-way ANOVA of the data (F (1, 45) = 1.498, P = 0.2274) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding time sent exploring the familiar object on PnD60, hence no post hoc analysis is allowed. The main factor effects of line of rat was also not significant (F (1, 45) = 0.01232, P = 0.9121). Whereas METH treatment did cause an increase in the amount of time spent exploring the familiar object in both rat strains relative to vehicle controls (F (1, 45) = 25.30, P < 0.0001).

In Figure 3-4D the two-way ANOVA of the data (F (1, 45) = 0.8329, P = 0.3663) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding time sent exploring the novel object on PnD60, hence no post hoc analysis is allowed. Both the main factor effects of line of rat (F (1, 45) = 1.379, P = 0.2464) and drug treatment (F (1, 45) = 0.2613, P = 0.6117) was not statistically significant.

It can be seen in Figure 3-4 that METH significantly increased the time spent exploring the familiar object in both FSL and FRL rats relative to saline-treated rats on PnD35 and PnD60. However, no statistical significance was found regarding time spent exploring the novel object.

3.4 Discussion

The data obtained from the FST (Figure 3-1) indicate that FSL rats were as a group more immobile than FRL rats, confirming the face validity of the FSL genetic rat model of depression. Furthermore, chronic pre-puberty administration of METH to FSL and FRL rats for 16 days induced antidepressant-like behaviour when tested immediately before withdrawal (PnD35), a response most likely associated with its psychostimulant effects. Other studies have similarly shown that METH causes a decrease in immobility time (antidepressant-like effects) in a dose dependant manner when tested directly after administration (Shimazu *et al.,* 2005). However, after 25 days withdrawal (PnD60) we show that the METH-induced antidepressant-like effect was reversed and, in fact, a depressogenic effect was observed in METH-treated FRL and FSL rats relative to vehicle-treated animals. It may be deduced that pre-puberty exposure of rats to METH results in long-lasting depressogenic effects, putatively via effects on neurodevelopment and a resulting effect on regional brain monoamines, as recently described (Strauss *et al.,* 2014). Previous studies have indicated that withdrawal from chronic amphetamine administration can result in behavioural alterations with characteristics similar to that of depression in humans (McGregor *et al.,* 2003), for example the elevation of the reward threshold in the brain with deficits in reward as a consequence (Cryan *et al.,* 2002). The data described here indicates a psychostimulant withdrawal-induced depression that is supported by earlier studies. This is a robust phenomenon that is detectable in numerous behavioural tests in rats, including the intra-cranial self-stimulation (ICSS) procedure, the FST as well as the tail suspension test (Cryan *et al.,* 2002). Psychostimulant-withdrawal-induced depression also seems to be long-lasting (McGregor *et al.*, 2003) and may be the result of the enduring neurotoxic effects of METH. In fact, in agreement with numerous reports that METH causes neuronal damage via increased oxidative stress (Tocharus *et al.,* 2010), a recent study in our laboratory demonstrated that chronic METH exposure induced a significant decrease in superoxide dismutase (SOD) activity as well as significantly increased lipid peroxidation in various brain regions of the rat (Strauss, 2012).

This long-lasting effect also seems to be additive to the congenital depressive-like phenotype in FSL rats, suggesting a role for genetic susceptibility. This observation is in line with a two-hit hypothesis of depression, suggesting that the manifestation of depression will result when a genetic predisposition is followed by an environmental stressor (i.e. METH) later in life. The data suggest as working hypothesis that individuals that already have a predisposition to depression may be more susceptible to developing depression when abusing METH early in life. This finding is also supported by similar data from previous studies in our laboratory using FSL rats (Swart, 2013). On the other hand, studies using

combined METH plus social isolation rearing (SIR) (Strauss *et al.,* 2014), thus a history of environmental adversity, did not support the two hit hypothesis. However, Strauss argues in favour of a ceiling effect that prevented more prominent neurobehavioral deficits from occurring (Strauss *et al.,* 2014). Similarly, METH administration, maternal separation and the combination of the two stressors decreased BDNF levels in both the dorsal and ventral hippocampus, while in the dorsal hippocampus, nerve growth factor (NGF) remained unaltered by either stressor alone or in combination. The lack of a greater combined effect prompted the authors to propose a possible compensatory mechanism in response to METH following maternal separation (Dimatelis *et al.,* 2014). That METH plus FSL supports the two-hit hypothesis whereas METH plus either maternal separation or social isolation does not, is of interest. It could be that METH fails to illicit a compensatory mechanism in FSL rats as it apparently does in the aforementioned two models. We would put forward the notion that the FSL plus METH model is arguably more suitable for studying the two-hit hypothesis, although further studies that investigate this phenomenon are needed.

Locomotor activity assessment (see Figure 3-2) indicated that METH treatment decreased locomotor activity in FSL and FRL rats compared to their vehicle controls on PnD35. However, on PnD60 METH did not affect locomotor activity in FSL or FRL rats compared to their vehicle controls. It has been shown previously that METH-treated animals are significantly less active (reduced locomotor activity) than their saline treated controls (Herring *et al.,* 2008; Wallace *et al.,* 1999; Swart, 2013). In addition METH-treated animals have displayed a smaller number of beam breaks than saline treated control animals (Herring *et al.,* 2008), thus similar to what we describe here. However, earlier work seems to suggest that assessment of repetitive beam breaks does not reveal evidence for METH-induced hyperactivity, although there is evidence for an increase in focused movement, possibly indicative of stereotypy (Herring *et al.,* 2008). Although repetitive beam breaks are not a measure of stereotypy, they do indicate that aspects of focused movements contribute to the spectrum of stereotypic behaviours observed in METH-treated animals (Herring *et al.,* 2008). This reduction in locomotion may be due to the decrease in extracellular DA generally observed after METH treatment, and is supportive of earlier work where the spontaneous activity of rats was found to be significantly decreased following treatment with a neurotoxic regimen of METH (Wallace *et al.,* 1999).

However, after wash-out, we found that animals did not display any significant differences in locomotion regardless of strain or treatment. Thus METH's ability to decrease locomotor activity seems to be dependent on the acute effects of METH to alter central monoamine levels (PnD35) and is not long-lasting after withdrawal (PnD60). Previous studies have

indicated that METH markedly increases locomotor activity initially, with a long-lasting enhancement of locomotor activity over a couple of hours (Wallace *et al.,* 1999) that was lost after metabolism of the drug (Wallace *et al.,* 1999). A previous study indicated that METH-treated animals exhibited lower activity during the initial exploratory phases (for the first 30 min) than saline-treated rats, although locomotor activity in both groups then reached comparable levels during the last 30 minutes of the trial. Thus both METH- and saline-treated animals showed similar levels of activity at the end of the test period (Wallace *et al.,* 1999).

It is important to note that the decrease in locomotor activity (hypo-activity) on PnD35 could not have contributed to the decreased immobility observed in the FST (rather the opposite), confirming that the decreased immobility in Figure 3-1A indeed reflects psychomotor and not locomotor effects (i.e. antidepressant-like activity). On PnD60, however, no significant differences in locomotor activity were observed before and after METH, or between FSL and FRL rat lines, so that METH-induced enhancement of immobility in Figure 3-1B indeed reflects psychomotor deficits akin to depression and not locomotor effects (i.e. long-lasting depressogenic-like activity of METH).

The data in Figure 3-3 A & B demonstrated that METH significantly lowers social interaction behaviour (staying together) in both FRL and FSL rats, both immediately following drug treatment (PnD35) and after withdrawal (PnD60). It is therefore clear that this effect of METH is acute *and* long-lasting, putatively related to neurodevelopmental effects that sustains its adverse neurological effects. These findings are in agreement with previous studies conducted in our laboratory (Strauss, 2012; Strauss *et al.,* 2014). Our data (Figure 3-3A & B), however, did not demonstrate any significant difference in social interaction between FSL and FRL rat lines. Decreased social interactive behaviour is characteristic of the FSL rat where it displays significant anxiogenic behaviour in the social interaction test. Pairs of FSL rats tend to avoid each other when passing rather than grooming or crawling over or under each other (Overstreet *et al.*, 2005). Previous studies have shown a decrease in frequency and duration of social interaction between a pair of unfamiliar METH-treated rats (Slamberova *et al.,* 2010; Strauss, 2012; Strauss *et al.,* 2014). However, Samberova (2010) and colleagues found that social interaction was increased after administration of lower doses of METH (1.25mg/kg) and decreased after higher doses of METH (2.5 – 5 mg/kg) (Slamberova *et al.,* 2010). Therefore, the fact that this study used an escalating dose regimen and consequently high doses of the drug could have contributed to the anti-social behaviour observed. Previous data has also indicated that when METH is administered in higher doses the rats display stereotypical behaviour, for example sitting in the corner of the

test box indulging in repetitive head movements with little to no social interaction (Slamberova *et al.,* 2010). Interestingly, the unfamiliar environment of the test arena seems to play a vital role in the social interactive behaviour observed. It has been documented that an unfamiliar environment increases time spent in explorative behaviour (locomotion) and therefore decreases the time that the rats stay together. The fact that no habituation trial was conducted in the SIT could have contributed to the decrease in time spent in social interactive behaviour.

Self-directed behaviour (self-grooming) was also assessed using the SIT, indicating no statistically significant interaction between line of rat and drug treatment neither on PnD35 nor on PnD60. However, METH treatment did decease self-grooming time significantly on PnD35. On PnD60 the main factor effect of line of rat did indicate significance, although drug treatment did not. METH-treated animals displayed a decrease in self-grooming behaviour (self-directed behaviour) on PnD35 (Figure 3-3C), however such a METH-induced decrease was not statistically significant after withdrawal on PnD60 (Figure 3-3D). An increase in self-directed behaviour was expected, since it has been documented in our laboratory before (Strauss, 2012) and elevated self-grooming may be indicative of increased anxiety (Moller *et al.,* 2011). Thus this study showed that METH treatment did not induce anxiety-like behaviour on PnD35, and did not affect self-grooming on PnD60.

Novel object recognition was altered in both the FSL and FRL rats in the nORT where METH treatment enhanced exploration of the familiar object before withdrawal at PnD35 (Figure 3-4A). It has been documented that if novel object recognition memory is intact, rats will spend more time exploring the novel object (Mathiasen & DiCamillo, 2010). Thus, the fact that the rats investigated the familiar object for a greater amount of time relative to controls may be the result of loss of recognition memory for the familiar object. When the rats do not recognize the familiar object, it is seen as a novel object and therefore the rats will spend more time investigating this object. This lack of recognition memory was found to be long-lasting yet still present after withdrawal at PnD60 (Figure 3-4C). This is a very interesting finding and the literature does not provide a clear explanation for this phenomenon. Nevertheless, it is well known that memory involves the laying down of long-term synaptic changes and events related to neuroplasticity that are also involved in neuronal development (Eriksson *et al.,* 2012). Thus, that compromised memory performance was observed immediately after METH exposure and after withdrawal is extremely important albeit not unexpected, indicating a sustained adverse effect on neurodevelopment. This study therefore represents the first time that this phenomenon has been described.

Different from what was seen regarding time spent exploring the ***familiar*** object above, METH treatment did not significantly affect the time spent exploring the ***novel*** object in FSL and FRL rats, either before withdrawal at PnD35 or after withdrawal at PnD60 (Figure 3-4B & D). These findings would suggest that recognition memory was indifferent between FSL and FRL rats, and that it is also not significantly affected by METH. Different from our findings, previous studies have reported that animals treated with METH spend less time exploring the novel object compared to their saline controls (Herring *et al.,* 2008; Reichel *et al.,* 2011). Interestingly, memory loss and cognitive deficits, which are symptoms of MDD, were not previously recognised as a behavioural characteristic of the FSL rat, and no definitive evidence of cognitive disturbances under basal conditions have been documented (Overstreet *et al.*, 2005). However, recent studies do indicate that cognitive impairments do indeed exist (Eriksson *et al.,* 2012). Abildgaard (2011) and colleagues found that the depressive-like phenotype of FSL rats is associated with impairment in novel object recognition memory (Abildgaard *et al.,* 2011). Furthermore, a study conducted in 2012 indicated that the FSL rat line can be employed in studies as a model of reversible impairments in emotional processing and memory. The FSL rat also displays predictive validity for evaluation of drugs supporting cognitive performance (Eriksson *et al.,* 2012). It has also been reported that lower doses of METH enhance memory and cognition, and thus neurotoxic doses can affect memory and cognition in a different way (Meneses *et al.*, 2011). It is therefore clear that there are conflicting findings in the literature in this regard. However, the rat line used and the ages at the time of analyses differed from previous studies. The current study performed behavioural analyses at PnD35 and PnD60, where previous studies only performed analyses on PnD60. This method allowed us to compare the effects of METH directly after the injection period as well as later in life, making the study more robust.

3.5 Conclusion

This study has demonstrated that chronic METH treatment produces significant behavioural effects directly after the injection period (PnD35) and later in life (PnD60). METH induced antidepressant-like behaviour before withdrawal in the FST, however, after 25 days withdrawal, METH-induced antidepressant-like effect was reversed and depressogenic effects were observed in METH-treated FRL and FSL rats. This long-lasting effect also seems to be additive to the congenital depressive-like phenotype of FSL rats, suggesting a role for genetic susceptibility. This observation would be in line with a two-hit hypothesis of depression, suggesting that the manifestation of depression will result when genetic predisposition is followed by an environmental stressor (i.e. METH) later in life. The data suggest as working hypothesis that individuals that already have a predisposition to depression may be more susceptible to developing depression when abusing METH. The

fact that the FSL rats were more immobile than FRL rats also confirmed the face validity of the FSL genetic rat model of depression. Locomotor activity assessment indicated that METH treatment decreased locomotor activity in FSL and FRL rats compared to their vehicle controls on PnD35. However, on PnD60 METH did not affect locomotor activity in FSL or FRL rats. It is important to note that the effects observed in locomotor activity could not have contributed to the immobility observed in the FST, confirming that the immobility in the FST indeed reflects psychomotor and not locomotor effects. The study also demonstrated that METH significantly lowers social interaction behaviour in both FRL and FSL rats, both immediately following drug treatment (PnD35) and after withdrawal (PnD60). It is therefore clear that this effect of METH is long-lasting, putatively related to neurodevelopmental effects. In addition the rats investigated the familiar object for a greater amount of time in the nORT on PnD35 and PnD60 and may be the result of loss of recognition memory for the familiar object. This data confirms that METH results in cognitive memory deficits probably due to sustained adverse neurodevelopmental effects.

Thus, early-life exposure to adversity, like drug abuse, may aid the development of late-life behavioural abnormalities and neurodegenerative diseases, in particular depression. The current study demonstrated that pre-adolescent exposure to METH can reproduce most of the behavioural changes seen in depressed individuals. Furthermore, although further study is required, the data suggests that early-life exposure to METH in predisposing individuals (male FSL rats) may culminate in behavioural abnormalities later in life, particularly a mood disorder.

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3.7 References

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Chapter 4 – Summary, final conclusions and recommendations

4.1. Summary of study outcomes

Since the results of the current study are presented in separate chapters i.e. Chapter 3 (Scientific Article) and Addendum B (Supplementary and Alternative Results), it is necessary to include a concise summary of the results to relate the behavioural effects of METH to alterations in the neurochemical markers observed (see Table 4-1).

This chapter represents the results obtained from a battery of behavioural test performed on PnD35 or PnD60 in pre-adolescent METH- or saline treated FSL and FRL rats. Thereafter, analyses of monoamines and their metabolites were performed. The methods regarding the specific behavioural tests as well as the neurochemical analyses can be found in Addendum A.

As defined in Chapter 1, the current study aimed to investigate long-term effects of early-life exposure to METH on:

- Depressive-like behaviour in the forced-swim test (FST);
- Social interactive behaviour in the social interaction test (SIT);
- Self-directed behaviour in the social interaction test (SIT);
- Cognitive function in the novel object recognition test (NORT);
- Locomotor activity in the Digiscan (AccuScan) apparatus;
- A possible link between the observed behavioural changes in the animals with monoamines levels and their metabolites in the frontal cortex.

Table 4-1: Summary of the behavioural tests and neurochemical analyses data that were performed on PnD35 and PnD60 were '↑' = increased, '↓' = decreased and '-' = no change.

Behavioural Tests	Control (Saline-treated) PnD35	Control (Saline-treated) PnD60	Treatment (METH-treated) PnD35	Treatment (METH-treated) PnD35	Treatment (METH-treated) PnD60	Treatment (METH-treated) PnD60
	FSL vs FRL	**FSL vs FRL**	**FRL** (vs FRL control)	**FSL** (vs FSL control)	**FRL** (vs FRL control)	**FSL** (vs FSL control)
Immobility (FST)	↑	↑	↓	↓	↑	↑
Locomotor activity (Digiscan®)	-	-	↓	↓	-	-
Staying together (SIT)	-	-	↓	↓	↓	↓
Self-grooming (SIT)	-	↓	↓	↓	-	-
Familiar object exploration (nORT)	-	-	↑	↑	↑	↑
Novel object exploration (nORT)	-	-	-	-	-	-

Neurochemical Analyses	Control (Saline-treated) PnD35	Control (Saline-treated) PnD60	Treatment (METH-treated) PnD35	Treatment (METH-treated) PnD35	Treatment (METH-treated) PnD60	Treatment (METH-treated) PnD60
	FSL vs FRL	**FSL vs FRL**	**FRL** (vs FRL control)	**FSL** (vs FSL control)	**FRL** (vs FRL control)	**FSL** (vs FSL control)
5-HT	-	↑	↓	↓	-	-
DA	-	↑	-	-	-	-
NE	-	↑	↓	↓	-	-
5-HIAA	-	↑	-	-	-	-
DOPAC	-	↑	-	-	-	-
HVA	-	-	-	-	-	-

4.2. Discussion of combined results

Neurochemical analyses indicated decreased 5-HT and NE levels on PnD35. METH is widely recognised for its pro-inflammatory effects (Tocharus *et al.,* 2010; Coelho-Santos *et al.,* 2012), and the reduced 5-HT levels observed may have been the result of an increase in circulating pro-inflammatory cytokines. Cytokines such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), induce indoleamine 2,3-dioxygenase (IDO) resulting in a shunting of tryptophan metabolism toward the production of kynurenine and quinolinic acid rather than 5-HT and thus decreasing 5-HT levels (see Figure 4-1) (Soskin *et al.,* 2012). Indeed interferon-γ is known to induce depression in humans (Dantzer *et al.,* 2008). Several studies implicate decreased serotonergic activity in major depression and acute trypotophan depletion studies support this theory. The neurotoxic effects of METH are linked to oxidative stress and it has been show that factors that induce oxidative stress evoke monoaminergic changes (Moller *et al.,* 2013) and can thus results in a decrease in 5-HT.

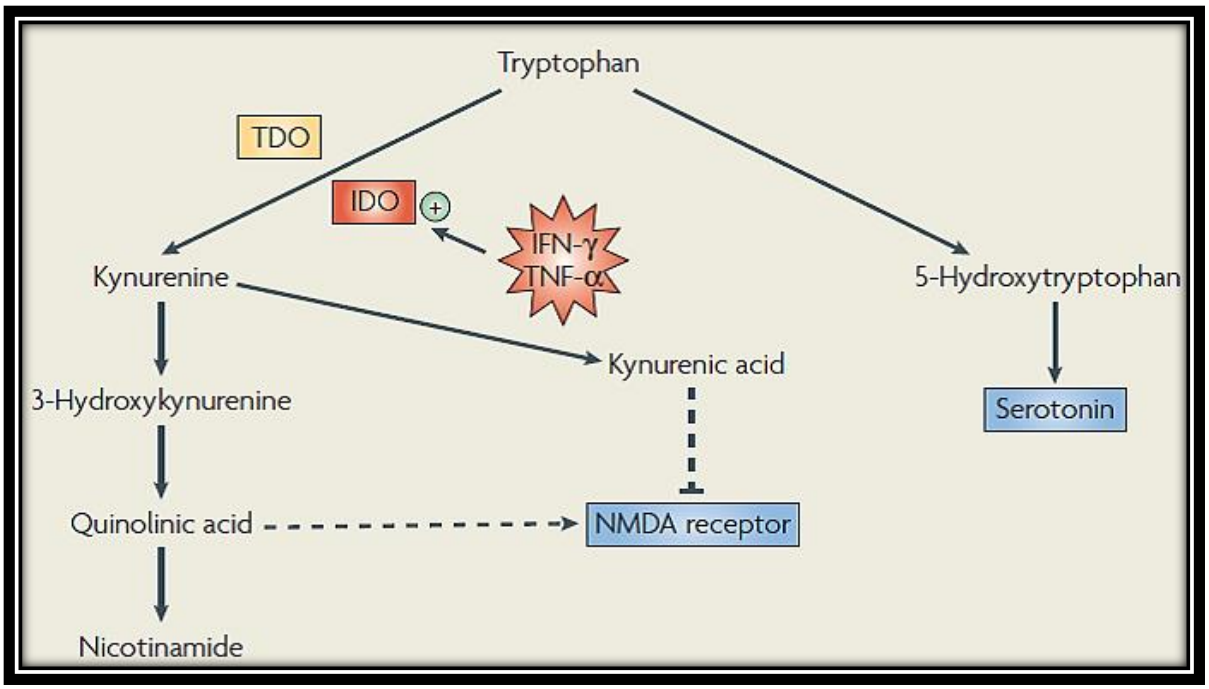

Figure 4-1: Indoleamine 2,3-dioxygenase (IDO) degrades tryptophan through the kynurenine pathway (Dantzer *et al.,* 2008).

According to the permissive hypothesis manic and depressive episodes are characterised by a decreased central 5-HT function (Spencer, 1977) where deficits in 5-HT can cause NE levels to fall below normal ranges which then results in depression (Hilty *et al.,* 2006). METH causes the rapid release of 5-HT and NE into the synapse and may thus disrupt the balance between these neurotransmitters resulting in mood disorders such as depression. Thus,

these observations are in line with the permissive hypothesis of depression and can be related to the depressive-like behavioural symptoms observed during behavioural testing. Although METH is known to cause perturbations in DA, this was not evident in this study. In fact, as will be discussed below, our data indicates that DA is less responsible for the behavioural effects evoked by METH, whereas altered 5-HT and NE would appear to have a distinct causal role.

METH induced antidepressant-like behaviour before withdrawal on PnD35 in the FST, which some literature suggests may be related to an increase in DA transmission in the mesolimbic pathway (Dipiro *et al.,* 2011). Such effects may also be responsible for METH's ability to induce intense euphoria. However, neurochemical analyses did not indicate a significant increase in DA on PnD35. It is vital to also consider 5-HT's effects on antidepressant behaviours. As noted above, METH was associated with a significant reduction in frontal cortical 5-HT on PnD35. Current thinking, especially those propagating the biogenic amine hypothesis and supported by the serotonergic actions of most antidepressants in clinical use at present, adhere to the belief that low 5-HT levels are associated with depression. This is not entirely valid. A newer antidepressant drug, tianeptine, is a selective serotonin reuptake enhancer (SSRE) yet is a clinically effective antidepressant drug. It therefor acts by *decreasing* 5-HT in the synapse which is in contrast to other antidepressant therapies (Malagié *et al.,* 2000). Thus, in line with the effects described for METH in this study, an anti-depressant effect can be achieved by lowering 5-HT levels. In addition, the new generation antidepressant, agomelatine, is a 5-HT_{2C} receptor antagonist does not release 5-HT, contrary to many current antidepressants (Harvey & Slabbert, 2014). Thus, the observed reduction in 5-HT could have caused the antidepressant effects seen in the FST (decreased immobility) on PnD35. According to a recent study, antidepressant (SSRI) discontinuation may result in an increase in 5-HT that can result in the antidepressant discontinuation syndrome and possibly drive subcellular mechanisms associated with relapse (Harvey & Slabbert, 2014). Such mechanisms include reduced neuroplasticity as well as increased glutamate-nitrergic signalling that are damaging to neurons (Harvey & Slabbert, 2014). In addition, mice that lack SERT function exhibit elevated extracellular 5-HT levels, that usually mimics antidepressant therapy, yet the animals display depressive-like behaviours (Ansorge *et al.,* 2007). Thus under certain conditions an increase in 5-HT levels may be counter-productive in the treatment of depression.

After 25 days of withdrawal, METH now induced a depressogenic effect in the FST in METH-treated FRL and FSL rats. The long-term usage of METH results in down-regulation of D_2 receptors and a decrease in the number of reuptake sites (Karila *et al.,* 2009). Hypo-activity

of DA cells is expressed in the form of reduced extracellular DA levels and explains the neural basis of the dysphoric state observed in METH abusing individuals during the withdrawal phase (Melis *et al.,* 2005). Unfortunately, the data from the current study did not indicate a change in frontal cortical DA levels on PnD60. This may indicate that changes in DA may have occurred but are not reflected by changes in its concentration. On the other hand, DA may have played a smaller role in the observed depressogenic effects induced by METH. Of interest is that frontal cortical 5-HT levels were *still* reduced on PnD60 in the FSL rats, albeit non-significantly. We would argue then that METH causes antidepressant effects immediately following chronic administration via decreasing 5-HT, whereas the long-term effects of METH post withdrawal are depressogenic by virtue of the sustained suppression of 5-HT that, according to the permissive hypothesis, "permits" the genesis of a mood disorder. However, altered serotonergic function modulates other neurotransmitters, including NE and glutamate (Harvey & Slabbert, 2014) so that a shift in any direction can result in alterations in monoamines giving rise to a mood disorder.

Interestingly, METH-induced damage to DA terminals has been reported to be more severe in the dorsal striatum in comparison with the nucleus accumbens and prefrontal cortex, which is said to be caused by the different densities of the DAT in these brain regions (Schwendt *et al.,* 2009). It is also documented that the adolescent brain, particularly the dopaminergic system, is relatively resistant to METH neurotoxicity (Killinger *et al.,* 2014) and previous studies have found on PnD20 that rats have fewer DAT binding sites than adult rats and are resistant to the neurotoxic effects of METH (Fumagalli *et al.,* 1998). Thus, the latter studies may explain why no significant changes were observed in DA levels (Thomas *et al.,* 2009). METH effects on 5-HT however, are distributed throughout the frontal cortex (Schwendt *et al.,* 2009) and are in line with our current data and the earlier explanations.

Data from the FST seems to be additive to the congenital depressive-like phenotype in FSL rats, suggesting a role for genetic susceptibility. This observation would be in line with a two-hit hypothesis of depression, suggesting that the manifestation of depression will result when genetic predisposition is followed by an environmental stressor (i.e. METH) later in life. The data suggest as working hypothesis that individuals that already have a predisposition to depression may be more susceptible to developing depression when abusing METH.

Locomotor activity assessment indicated that METH treatment decreased the locomotor activity compared to their vehicle controls on PnD35. However, on PnD60 METH did not affect locomotor activity in FSL or FRL rats compared to their vehicle controls. This reduction in locomotion is normally due to the decrease in extracellular DA after METH treatment, and

is supportive of earlier work where the spontaneous activity of rats was found to be significantly decreased following treatment with a neurotoxic regimen of METH (Wallace *et al.,* 1999). However, after wash-out, the animals did not display any significant differences in locomotion regardless of strain or treatment. The reduction in locomotor activity on PnD35 can be attributed to the decreased 5-HT and NE observed. Previous studies have found that depletion of cerebral 5-HT levels results in reduced physical activity and consequently the development of obesity (Uceyler *et al.,* 2010). In addition, acute or chronic inactivation of the NET reduces spontaneous locomotor activity in both novel and familiar environments (Mitchell *et al.,* 2006). Our data also indicates no significant changes in 5-HT and NE on PnD60 which may explain why no significant deficits in locomotor activity were observed after withdrawal. Thus, METH's ability to decrease locomotor activity seems to be dependent on the acute effects of METH to alter central monoamine levels (PnD35) and is not long-lasting after withdrawal (PnD60).

The data demonstrated that METH significantly lowers social interaction behaviour (staying together) in both FRL and FSL rats relative to vehicle controls, both immediately following drug treatment (PnD35) and after withdrawal (PnD60). It is therefore clear that this effect of METH is long-lasting, putatively related to neurodevelopmental effects. This phenomenon was first documented in 2004 where long-term decreases in social interaction were observed following METH administration (Clemens *et al.,* 2004). Social withdrawal is characteristic of depression and is regarded as a diagnostic feature of depression.

Object recognition was altered in both the FSL and FRL rats in the nORT where METH treatment enhanced exploration of the familiar object before withdrawal at PnD35. This effect was long-lasting and still present after withdrawal at PnD60. If novel object recognition memory is intact the rats will spend more time exploring the novel object (Mathiasen & DiCamillo, 2010). Thus, the fact that the rats investigated the familiar object for a greater amount of time relative to controls may be the result of loss of recognition memory for the familiar object. When rats do not recognize the familiar object, it is seen as a novel object and therefore the rat will spend more time investigating this object. Previous studies have reported that damage to or loss of DA neurons occurs after exposure to METH (Panenka *et al.,* 2012) and may thus explain why impaired cognition was observed in the nORT. A decrease in striatal DAT levels have also been reported in METH abusers, which is believed to be associated with psychiatric manifestations, as well as memory and motor deficits (Riddle *et al.,* 2006). DA is thought to be an essential modulator for prefrontal cortex (PFC)-mediated working memory tasks. Although no significant decreases were observed in DA, the loss of this neuromodulator causes definite cognitive impairments as seen in our study

and is also evident in patients that have been diagnosed with dementia (Stern & Alberini, 2013). Extensive research indicates that 5-HT and NE modulates memory function (Celikyurt *et al.,* 2012) and it has been documented that hippocampal-based memory deficits are reversed by SSRI's such as paroxetine which promotes neurogenesis in the hippocampus (Bremner, 2006). Thus a decrease in 5-HT could have resulted in the memory deficits observed in this study. Looking beyond the role of monoamines, since oxidative stress is associated with significant disturbances in monoamines (Mokoena *et al.,* 2014), and that METH induces cerebral oxidative stress (Strauss, 2012; Tocharus *et al.,* 2010), it could also be hypothesised that METH-induced oxidative stress could have resulted in memory deficits via secondary changes in monoamines via alteration in cellular redox status.

METH treatment significantly decreased 5-HT levels relative to controls on PnD35 but not on PnD60. Chronic use of METH causes depletion of monoamines and can thus explain why a decrease in 5-HT was observed directly after the injection period. Serotonergic pathways integrate sensory processing, cognition, emotion regulation and motor activity (Lesch, 2007), while serotonergic pathways are implicated in the hallucinogenic effects of 5-HT antagonists. The latter may explain why deficits were seen in the nORT where the rats explored the familiar object for a longer period of time as well as deficits in locomotor activity on PnD35 as well as social withdrawal. 5-HT is also vital in early brain development and adult neuroplasticity that includes cell proliferation, differentiation and synaptogenesis (Lesch, 2007). Moreover 5-HT modulates emotional behaviour including stress response, impulsivity, anxiety and aggression and underlies hallucinations associated with drug abuse (Ebbitt, 2008). Thus alteration in the serotonergic transmission early in life may produce deficits in emotional processes later in life.

4.3. Conclusion

In conclusion, chronic METH treatment during pre-adolescence induces significant behavioural changes related to depression in humans, such as deficits in cognition, decreased social interaction as well as decreased locomotor activity on PnD35. The animals also displayed antidepressant-like behaviour before withdrawal, yet a depressogenic effect was observed 25 days post-withdrawal. This long-lasting effect also seems to be additive to the congenital depressive-like phenotype in FSL rats, suggesting a role for genetic susceptibility. This observation would be in line with a two-hit hypothesis of depression, suggesting that the manifestation of depression will result when genetic predisposition is followed by an environmental stressor (i.e. METH) later in life. The data suggest as working hypothesis that individuals that already have a predisposition to depression may be more susceptible to developing depression when abusing METH.

Neurochemical analyses provided thought-provoking data concerning the role of the permissive hypotheses of depression, indicating that DA is most likely not responsible for the behavioural effects observed, at least under the current study conditions, whereas 5-HT is decidedly more involved than was expected. The data also suggest that depletion in NE plays a role in the development of depressive-like behaviours following METH exposure. Based on these findings, we propose that disturbances in 5-HT and NE are a crucial mechanism in how METH abuse may precipitate or worsen depressive-like symptoms in individuals who abuse METH. It should be noted that this study does not discard the role of DA in the development of depression after METH exposure, although under the current study conditions it appears that DA does not play a central role.

A significant decrease in 5-HT and NE in the frontal cortex was observed that may demonstrate the depletions of presynaptic vesicle stores. The fact that emotional behaviour is controlled through a delicate balance between NE and 5-HT levels and manic and depressive episodes are characterised by a decreased central 5-HT function (Spencer, 1977). Thus, deficits in 5-HT can cause NE levels to fall below normal ranges (Hilty *et al.,* 2006). METH-induced depletion of 5-HT may result in disruptions in the balance between these neurotransmitters resulting in mood disorders such as depression.

The current study demonstrated that pre-adolescent exposure to METH can reproduce most of the behavioural changes seen in depressed individuals, and that these behavioural data can be used to identify causal neurochemical factors. Environmental stressors such as METH abuse should be regarded as an additional diagnostic criterion and is relevant to an accumulative risk factor hypothesis. Furthermore, although further study is required, the data suggests that early-life exposure to METH may predispose an individual to mood disorders and behavioural abnormalities later in life.

4.4. Recommendations

The current study successfully addressed the objectives that were outlined in Chapter 1. However, there were some limitations to the study and thus recommendations are made for future studies:

1. Injection stress may have affected the results of the study despite the fact that injection stress were controlled and minimised by injecting the animals subcutaneously. The rats were injected twice daily for 16 consecutive days. Future studies may consider alternative routes of administration.

2. Future studies can also consider measuring additional neurochemical markers such as markers of oxidative stress, superoxide dismutase (SOD), glutamate signalling and lipid peroxidation. In addition neuro-inflammatory markers (cytokines) such as TNF-α, INF-α, BDNF and IL-6 should be determined. This tactic may provide greater insight on the neurotoxic effects of METH.

3. Measuring single-nucleotide polymorphisms (SNP's) in METH-treated FSL rats may also provide new insights to reproducibility among genetic association studies of depression since hippocampal dysregulation of neuropeptide Y (NPY) may be caused by these functional SNP's.

4. This study focussed on behavioural and neurochemical testing n PnD35 and PnD60, however, testing beyond PnD60 should be considered to determine long-lasting effects.

5. Investigating the effects of METH on alternative developmental stages such as the child or adolescent stage may be useful to determine and compare effects of different age groups to evaluate age-related susceptibility to METH.

6. The current study aimed to investigate the effects of early-life administrations of METH on late-life behavioural and neurochemical irregularities, however future studies should investigate the effects of anti-depressant agents after METH exposure to determine specific and effective treatment approaches and therapies.

Addendum A - Materials and Methods

A.1 Animals

This study, including all animal protocols, were approved in accordance with the regulations set by the Animcare Animal Research Ethics Committee of the North-West University (ethics approval no. NWU-000105-11-S5 – Approved: 07/10/2011). All experiments conformed to the guidelines of the National Institutes of Health for the care and use of laboratory animals. The handling of animals was performed in accordance with the guidelines for the use of animals in experimental work at the North-West University. All animals were maintained according to a code of ethics in research, training and testing of drugs in South Africa and complied with national legislation.

Male and female Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats were supplied by the Vivarium at the Pre-Clinical Drug Development Platform (PCDDP) of the North-West University. Animals were housed and bred at the Vivarium of North-West University (NWU), with the original colony obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA. The validation of the FSL rat as an animal model of depression and its unique characteristics are discussed in detail in Chapter 2. Although only the male rats were used for testing in the study, the female rats were used for breeding and nurturing the pups before weaning.

A.2 Drug Treatment

In the current study rats received subcutaneous administrations with methamphetamine hydrochloride (METH) and saline (vehicle), as indicated. METH was obtained from Sigma-Aldrich International GmbH (Sigma-Aldrich Manufacturing LLC, 3050 Spruce Street, St Louis MO 63103, United States of America) and thoroughly documented, stored, dispensed and used as prescribed/allowed by legislation. Once the METH was obtained it was stored in a dry locked safe that is protected from sunlight. A schedule 6 logbook was kept strenuously where the amount of drug removed, the date of removal and purpose of use were noted and signed. The safe and the logbook are managed under the supervision of a responsible registered pharmacist. METH crystals were dissolved in isotonic saline (METH-HCL) that served as vehicle to facilitate administration to the animals. The chemical and pharmacological properties of METH and METH-HCL are discussed in Chapter 2.

A.3 Administration

Chronic dosing of METH and saline was performed twice daily at 09:00 and at 15:00 for 16 consecutive days. The animals received a sub-cutaneous (SC) escalating dose regimen during the 16 day period, as indicated in Table A-1 below. The SC route of administrations was chosen because it minimizes handling and injection stress. A lipophilic drug such as METH is quite easily absorbed into the plasma after SC administration. This route of administration also assures accurate dosing and minimizes the risk of injuring the animals during an extensive administration period. This method also eases administration in rat pups and smaller rats.

Table A-1: Escalating dosage regimen for METH treatment over a period of 16 days from PnD19 to PnD34. The 1st dose was administered at 09:00 and the 2nd dose at 15:00 daily.

Day	1st Dose (mg/kg)	2nd Dose (mg/kg)
1	0.2	0.4
2	0.6	0.8
3	1.0	1.2
4	1.4	1.6
5	1.8	2.0
6	2.2	2.4
7	2.6	2.8
8	3.0	3.2
9	3.4	3.6
10	3.8	4.0
11	4.2	4.4
12	4.6	4.8
13	5.0	5.2
14	5.4	5.6
15	5.8	6.0
16	6.0	6.0

Study groups were injected twice daily with escalating doses (0.2 - 6.0 mg/kg increased with 0.2 mg/kg per administration) of METH or a saline control. METH administration was performed according to the body weight of the animals on that particular day, thus the animals were weighed before injection and thereafter the specific dose was calculated per individual rat. The escalating dose regimen was chosen specifically to mimic abuse in humans. METH abusers gradually increase the dose of the drug before high-dose exposure. Thus, in line with the trend of abuse in humans, the administration of METH followed a similar pattern.

The study included one early-life developmental stage, namely the "pre-adolescent phase", from PnD19 until PnD34 (sexual maturity reached on PnD35). The pups were randomly divided into 8 different treatment groups as depicted in Figure A-1 below on PnD19.

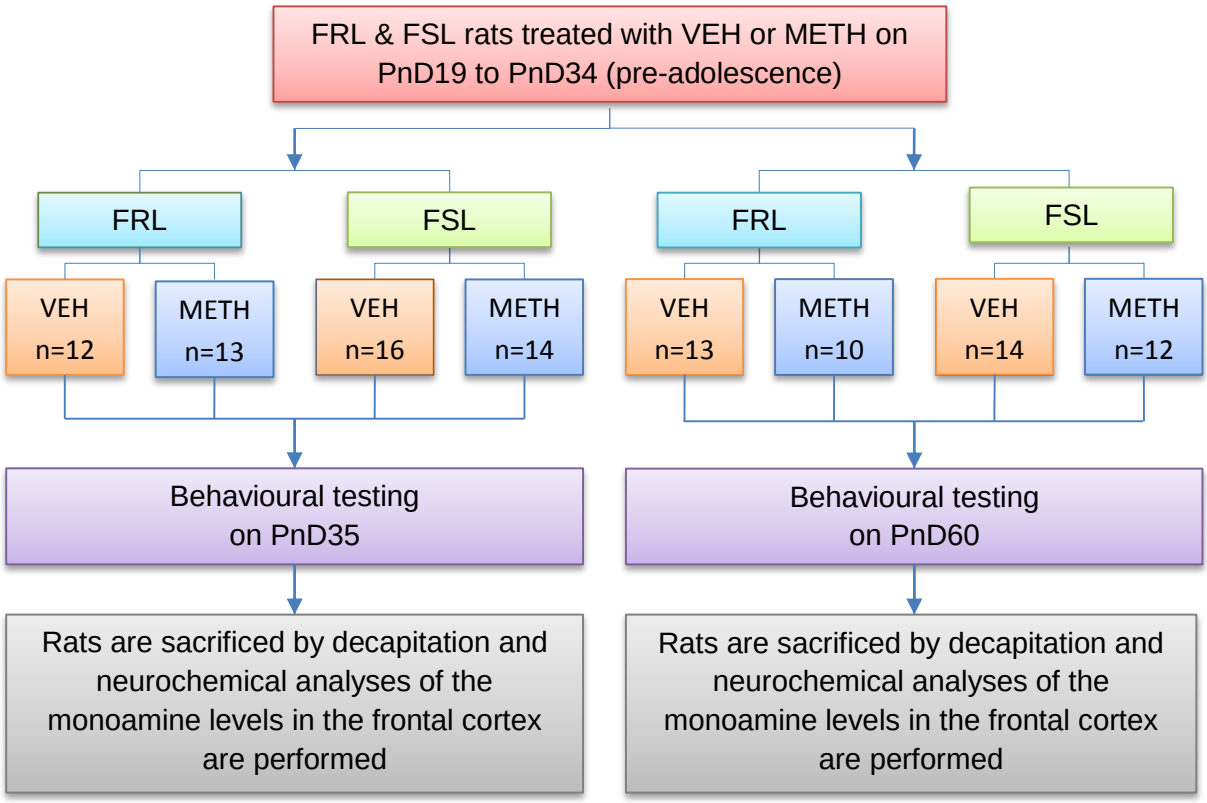

Figure A-1: Study layout of the various treatment groups, differing regarding the rat line, the treatment received and the postnatal day of behavioural testing. The number of animals per treatment group is also indicated. FSL = Flinders Sensitive Line rat; FRL = Flinders Resistant Line rat; VEH = vehicle control; METH = methamphetamine HCL.

As can be seen in Figure A-1, the treatment groups contained between 10 and 16 rats per group, depending on the birth rates. FSL or FRL rats received either vehicle or METH for 16 days until PnD34, where after rats were evaluated for immediate effects on PnD35, or for long-lasting effects after wash-out on PnD60 (early adulthood). The morning after behavioural tests concluded the rats were sacrificed by decapitation and whole brains were removed and dissected. The frontal cortex was removed and snap frozen in liquid nitrogen until the day of neurochemical analyses. Monoamines as well as their metabolites served as biomarkers to the study. The levels of these biomarkers were measured using the performance liquid chromatography system with electrochemical detection HPLC-EC.

A.4 Housing and Breeding Protocols in the Vivarium

The animals were housed under conditions of constant temperature (22 ± 1°C) and humidity (50 ± 10%) with a 12:12-h light/dark cycle (lights on at 06:00 and lights off at 18:00). Food and water were provided *ad libitum*. The animals were placed in new, clean cages weekly with fresh bedding (sawdust). No more than 4 animals were housed per cage. All cages were identical.

According to the breeding protocols a (one) FSL male rat was placed with a (one) female FSL rat for two consecutive nights. The following morning the male was removed from the cage and the female was assumed pregnant. The same procedure was followed for the FRL rat strain. Dams were normally housed during the gestational period and new-born pups were normally reared until weaning on postnatal day 21 (PnD21). Only male pups were included in the study and drug administration commenced on PnD19.

A.5 Behavioural Studies

Several of the characteristics of depression in humans have behavioural correlates in animals. These include lack of motivation, anhedonia, social withdrawal as well as cognitive abnormalities. Well-described, validated behavioural tests in animals usually measure only one of these behavioural links, thus a battery of behavioural tests are implemented to observe an array of behavioural correlates with major depression. The following behavioural tests were implemented in the current study: the novel object recognition test (nORT) to evaluate cognitive functioning (memory), the social interaction test (SIT) to evaluate anxiety-like behaviour, the Digiscan animal activity monitor to evaluate locomotor activity of the animals and the forced swim test (FST) to evaluate depressive-like behaviour. These tests were conducted in the sequence as described above in order to perform the most stressful behavioural test last i.e. the FST. All apparatus for the above mentioned tests were designed according to specifications described in literature and were manufactured by the Instrument Makers at the Potchefstroom Campus of the North-West University. Digital video cameras were mounted over the behavioural test apparatus to record events.

After the 16-day period of drug administration, the study investigated the behavioural effects of such treatment, either directly after the injection period on PnD35, or later in life after drug wash-out on PnD60, as explained above. Behavioural testing commenced after the start of the dark (active) cycle, between 18:00 and 00:00 and was conducted in a temperature controlled and soundproof room.

It has been shown that the order in which the behavioural tests are conducted can cause order effects when a test battery is performed (Blokland *et al.,* 2012). The FST also seems to be the most sensitive to test order and the results of this test rely on the order in which the behavioural tests were conducted (Blokland *et al.,* 2012). In this regard, a battery of tests were validated in our laboratory, comprising of, firstly, the novel object recognition test, followed by the social interaction test, then the locomotor activity test, thereafter the elevated plus-maze and lastly the forced swim test. Researchers in our laboratory were able to demonstrate that foregoing tests did not affect the outcome of the subsequent tests (Mokoena *et al.,* 2014). Thus, all four behavioural tests were conducted as a battery of tests for all treatment groups. In addition, periods of thirty minutes were permitted between tests to allow for habituation before the subsequent test and reduce any interference between different behavioural tests (Blokland *et al.,* 2012).

A.5.1 Novel Object Recognition Test (nORT)

The novel object recognition test (nORT) is used to evaluate declarative memory in rodents. This test is well described and validated and has been employed in numerous studies employing rodents. The nORT is a relatively high-throughput and robust, yet sensitive procedure that allows for evaluating compounds for cognition-enhancing activity (Mathiasen & Dicamillo, 2010). Thus this test may be used to assess the effects of a compound (METH) on the short-term memory of rats (Mathiasen & Dicamillo, 2010). This test relies on the principle that rats prefer to explore a novel (new) object relative to a familiar one. Rats are firstly exposed to two similar objects in an enclosed, familiar environment, and then following a 90 minute interval, they are exposed to a familiar object plus a novel object in the same familiar environment. With intact memory, the rat will then spend more time exploring the novel object rather than the familiar object, whereas equal time exploring the novel and familiar objects is indicative of impaired memory. Since impaired memory and cognition is a well-described characteristic of MDD (Ennaceur & Delacour, 1988), the nORT is useful to evaluate this aspect in rodents in conjunction with testing of depressive-like behaviour.

Dopamine (DA) is a well-known neuromodulator that affects cognition and the loss of this neuromodulator causes definite cognitive impairments. This is evident in patients that have been diagnosed with dementia (Stern & Alberini, 2013). A decrease in striatal DA transporter (DAT) levels have also been reported in METH abusers, which is believed to be associated with psychiatric manifestations, as well as memory and motor deficits (Riddle *et al.,* 2006). Thus, an understanding of both the short- and long-term effects of METH on striatal dopaminergic markers is crucial. Dopamine is thought to be an essential modulator for prefrontal cortex (PFC)-mediated working memory tasks. Decreased levels of dopamine

receptor (D_1) antagonists enhance working memory; this effect is revealed by an increase in neuronal activity (Stern & Alberini, 2013). Previous studies have reported that damage to or loss of dopamine (DA) neurons occurs after exposure to METH (Panenka *et al.,* 2012). Long-term METH use has also been associated with more severe psychiatric symptoms including paranoia, hallucinations and delusions. This finding may be attributed to a substantial reduction in DAT density in the brain (Smith *et al.,* 2012). However, serotonergic pathways integrate sensory processing, cognition, emotion regulation and motor activity (Lesch, 2007), and thus 5-HT deficiency could also be responsible for memory deficits.

The apparatus required for this procedure consists of a box made of opaque, black perspex, and with dimensions 500 mm x 500 mm, and a 400 mm surrounding wall. Two heavy objects, which cannot be moved by the rat, are placed inside the box, as indicated in Figure A-2.

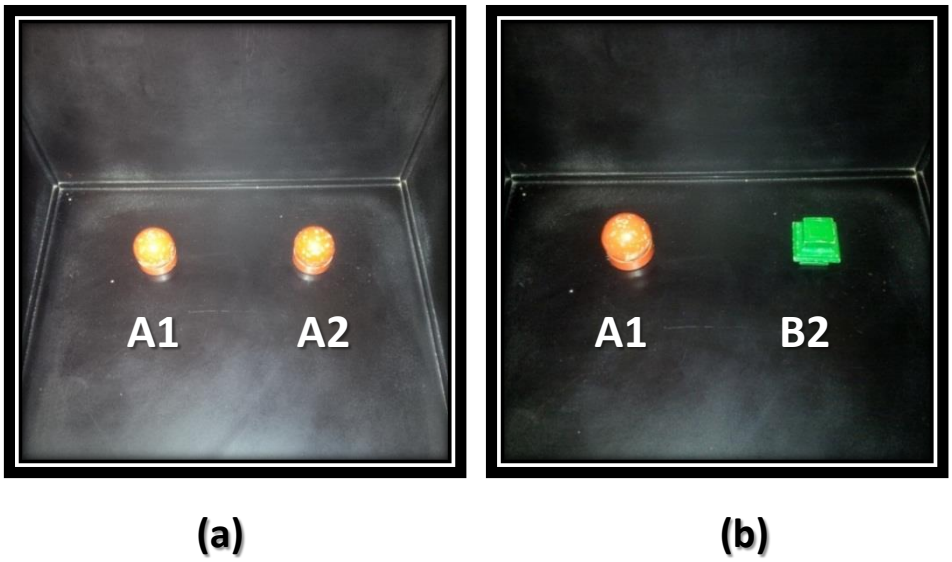

Figure A-2: The novel object recognition test apparatus for measuring acquisition memory. (a) Represents the setup for the acquisition trial where two identical objects (A1 and A2) are placed in the box as indicated. (b) Represents the setup for the retention trial where two dissimilar objects, namely one familiar object (A1) and one novel object (B2) are placed in the box as indicated. Dimensions of the test box are 500 mm x 500 mm x 400 mm (height).

The procedure of the test is as follows: The habituation trial commences two days prior to the night of testing (acquisition & retention trials), with the first habituation 48 hours before testing and the second 24 hours before testing. During the habituation trial the rats are given the opportunity to explore the empty nORT boxes (one rat per box) for 10 minutes. This trial allows the animals to familiarize themselves with the testing environment without

any objects. Thereafter, on the day of testing, the acquisition trial is conducted. The rats are given the opportunity to explore two identical, steady objects (A1 and A2) in the nORT box for a period of five minutes. These objects are identical in shape, size and colour as can be seen in Figure A-2: (a), and placed 10cm from the box walls in two adjacent corners. The rats are then returned to their home cages for a 1.5 hour inter-trial interval (90 minutes). The nORT box and objects are cleaned with 10% ethanol to remove any olfactory cues. The animals are then subjected to the last trial, named the retention trial. The rat is returned to the test box and presented with two steady objects to explore. One object will be the identical to the object in the first exploration trial (A1) while the other object will be a novel object (B2) as depicted in Figure A-2: (b). The new (novel) object has a different shape, size and colour and is therefore unfamiliar to the rat. Again the rats are allowed to explore the objects for five minutes, where after the rat is removed. Object exploration was defined as sniffing, licking or touching the objects with forepaws (Grayson *et al.,* 2007). Sitting near the object was not considered exploration. The exploration time (seconds) of each object in each trial was scored manually from the recorded videos using two stopwatches.

A.5.2 DigiScan (Locomotor Activity)

The DigiScan is an instrument used to measure locomotor activity. The Digiscan apparatus is depicted in Figure A-3 below.

Figure A-3: Digiscan behavioural test apparatus for the measurement of locomotor activity. The box contains infrared light beams that register any movements of the rat within the box.

The evaluation of locomotor activity is crucial for the interpretation of immobility in the FST (see discussion on the FST below), and for this purpose the Digiscan is a suitable low-stress

test. Previous studies suggested significant METH-induced locomotor hyperactivity and behavioural sensitisation (Kubota *et al.,* 2002).

The Digiscan (Accuscan Instruments) uses various infrared sensors placed strategically around the box to detect and measure activity within the cage (Cao *et al.,* 2002). Each open field monitor consists of sets of 16 light beams arrays in the horizontal X and Y axes. The 16 infrared light beams that traverse the animal monitor and can be arranged in an unlimited number of configurations. With the light-beam frames various parameters of the locomotor activity of the animals are measured. Within the square-shaped Digiscan apparatus, infrared sensors detect both the activity levels of the rats and the location where the activity was displayed. The rat is placed in the centre of the chamber and consequently the horizontal activity, vertical activity (rearings) as well as the total distance travelled by the animal is measured for five minutes. Activity is measured by recording the number beam breaks caused by the animal.

A.5.3 Social interaction test (SIT)

Depression is often characterised by social withdrawal behaviour. Impairments in social behaviours and a deficiency in social interaction skills are typically reported in patients suffering from depression. As a result patients are unable to integrate into the society.

The SIT is used to measure explorative and social interactive behaviours between pairs of rats in an open flied test (OFT) arena. The apparatus consists of a black square (100 cm x 100 cm x 40 cm) arena with opaque walls. The floor is divided into 25 cm x 25 cm equal squares (Sherif & Oreland, 1995) as seen in Figure A-4 below. The arena was illuminated with red light, with a video camera placed above the arena. When unfamiliar rats are tested in an unfamiliar arena with low levels of aversive stimuli, the test can be used to assess explorative and non-aggressive social behaviour, whereupon the model becomes very sensitive to drug-induced disturbances of normal explorative and social behaviours, e.g. antipsychotics (Moller *et al.,* 2011).

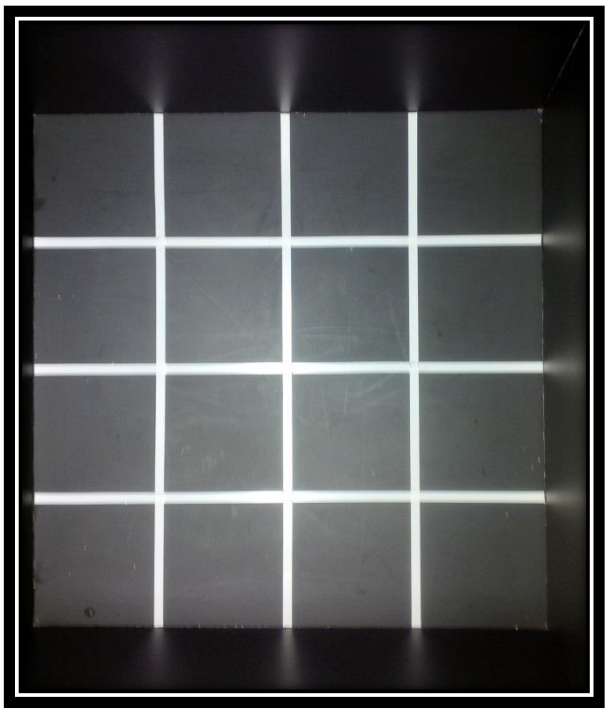

Figure A-4: The open flied test (OFT) apparatus used to asses social interactive behaviours. The floor of the arena is divided into squares using white lines.

A pair of unfamiliar rats was placed in the arena together. The pair of rats should not differ in weight by more than 10 grams, should be from the same rat line (i.e. FSL or FRL) and received the same treatment (VEH-or METH-treated). The time spent in active social interactive behaviours, self-directed behaviours and explorative behaviours are then scored for a period of 10 minutes. Active social interactive behaviour include anogenital sniffing, approaching and staying together (Moller *et al.,* 2011), self-directed behaviour include squares crossed and self-grooming and explorative behaviour include rearing (Moller et al, 2011). The researcher remained outside the testing room during the 10 minute testing period to avoid distraction. The arena was cleaned with 10 % ethanol solution between tests to eliminate olfactory cues from foregoing test animals (Ferdman *et al.,* 2007).

Due to the fact that a battery of behavioural test are conducted on the rats, the researchers decided to only score “staying together” (social interactive behaviour) and “self-grooming” (self-directed behaviour) for analyses in the SIT. Staying together is scored as one entity for both rats whereas self-grooming is scored for each individual rat.

A.5.4 Forced Swim Test (FST)

The FST is used to assess depressive-like behaviour in rodents (Abildgaard *et al.,* 2011; Blokland *et al.,* 2012). This test essentially determines learned helplessness behaviour which is a typical behavioural manifestation in depression. The test is based on the observation that rats placed in an inescapable cylinder of water and following initial escape-oriented movements, develop an immobile posture (Cryan *et al.,* 2002).

The FST is regarded as a screening test for antidepressant activity or depressive-like behaviour, and is not a translational model of depression. Although the immobility behaviour of the animals is caused by the test itself and is a direct reaction to the test procedure, the immobility does not persist after the test has concluded or outside the test situation. The FST does not cause an induction of a depressive state, however there are some components of construct validity i.e. stress-inducing conditions as well as a deceased behavioural output (Castagné *et al.,* 2009).

The rats were individually placed in a water-filled (25˚C ±2˚C) transparent plastic cylinder from which they cannot escape (height x 50 cm ; diameter x 25 cm ; water depth x 30 cm) as seen in Figure A-5. Upon exposure to the water for a period of 7 minutes, stress sensitive animals will almost immediately take on an immobile posture. Video monitoring was used to record the behaviour of the animals. Increased immobility, i.e. floating with minimal movements to keep the head above the water, during the FST has been proposed as a key element of depressive-like behaviour in rodents.

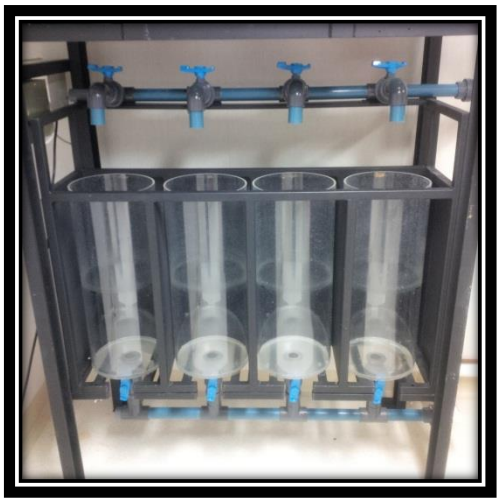

Figure A-5: The FST apparatus indicating the four individual cylinders.

The following activities were noted and scored. All three activities have been shown to reflect depressive-like behaviour and are depicted in Figure A-6.

- Immobility: assuming an immobile position, floating, no movements other than to keep their nose or mouths above the water surface to breath (Blokland *et al.,* 2012).
- Swimming: actively making swimming movements, more than those needed to keep their head above water (Blokland *et al*., 2012), across the surface of the water and moving around in the cylinder while maintaining a horizontal body position.
- Climbing: upward movements with the front paws in and out of the water surface, clawing against the side of the cylinder in an attempt to escape (Blokland *et al.,* 2012).

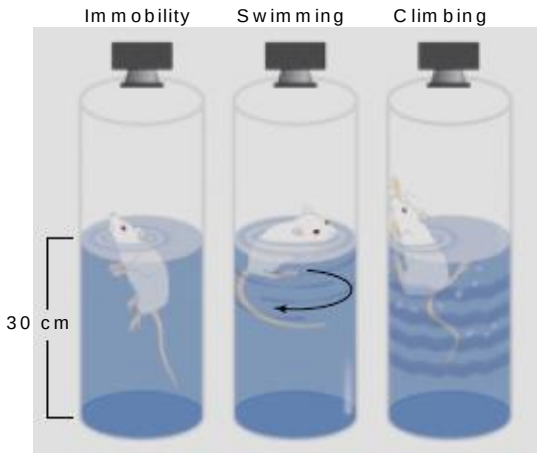

Figure A-6: The behavioural parameters measured in the modified forced swim test: Immobility, swimming and climbing behaviour (Cryan *et al.,* 2002).

Although the rats are placed in the cylinder for seven minutes, the first and last minute will not be scored to provide reliable full five minute period of scoring. The exclusion of the first minute in the water also excludes any initial anxiety-driven behaviour. Typically at some point during evaluation, the rat assumes an immobile position in order to conserve its energy as it will notice that an escape is impossible, a response similar to 'learned behavioural despair'. This is interpreted as giving up hope (Castagne *et al.,* 2009, 2010; Cryan *et al.,* 2002). Thus, antidepressants normally decrease the duration of immobility observed (Castagné *et al.,* 2009). Other activities, for example swimming and climbing, will also be measured to increase the sensitivity of the test, especially when used to access drug response. An increase in climbing activity denotes a prominent adrenergic response, while an increase in swimming activity denotes a prominent serotonergic response (Cryan *et al.,* 2002). The water was changes between each trial.

A.6 Neurochemical Studies

The current addendum also contains additional and supplementary information concerning the material and methods that were used during neurochemical studies including the methodology and experimental procedures in the preparation of the brain tissue and the measurements of monoamine levels using the high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC).

A.6.1 Regional brain monoamine analysis

A.6.1.1 Introduction

After the 16-day injection period concluded, the study investigated the neurobiological effects of such treatment directly after the injection period on post-natal day 35 (PnD35) as well as later in life on post-natal day 60 (PnD60). The morning after behavioural tests concluded the rats were sacrificed by decapitation without any prior use of an anaesthetic agent. Whole brains were removed and placed on an ice-cooled slab, thereafter, the frontal cortex was dissected out, placed individually into polyproylene tubes, marked and immediately snap frozen in liquid nitrogen and stored at -86°C until the day of neurochemical analyses. The brain tissue collected was then used for regional quantification of monoamines as well as their metabolites that served as biomarkers to the study. Quantification of cortico-striatal DA, 5-HT, NE, homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) were performed using the performance liquid chromatography system with electrochemical detection (HPLC-EC), as previously described (Harvey *et al.,* 2006; Moller *et al.,* 2013). This method has been validated with regards to stability, linearity, precision and accuracy as well as recovery in our laboratory (data in preparation for separate publication). The methodology is listed below.

Total brain monoamine levels and their metabolites served as the biomarkers to this study. Each individual frontal cortex from each rat was measured separately, however data from rats in the same group were combined. The concentrations of these biomarkers in each individual brain sample were determined by calculating the area under the curve (AUC) for each sample to that of the internal standard (isoprenaline) and to the monoamine standard (range 2.5 ng/ml - 50 ng/ml; Chromeleon® Chromatography Management System version 6.8. data acquisition and analysis software). DA, 5-HT and NE as well as their metabolites (5-HIAA, DOPAC and HVA) are expressed in ng/mg wet weight of brain tissue. The HPLC-EC analyses were performed by myself, under the guidance of Mr. Francois Viljoen (Scientific Officer – NWU).

A.6.2 Materials and methods

A.6.2.1 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: Kinetix C18 column, 150 x 2.1 mm, 2.6µm, 100 Å pores, (Phenomenex, Torrance, CA).

Mobile Phase: 0.1 M Sodium formate buffer, 0.5 mM ethylene-diaminetetraacetic acid (EDTA dinatriumsalt Na2EDTA), 5 mM sodium heptane sulphonic acid, 55ml acetonitrile and 15ml methanol. The pH of the mobile phase was set at ± pH 3.82 with orthophosphoric acid (10%).

NOTE: **This method is highly pH sensitive.**

Flow rate: 0.20 ml/min.

Injection volume: 20 µl

ECD Detector settings: ESA 5011A Analytical Cell Potential settings,
Volts: E1: +150mV
E2: +750mV
Gainrange: 20nA,
Polarity: Positive,
Reaction: Oxidation,
Guardcell Potential setting: +150mV

Mini Validation data – System Suitability:

Linearity / Calibration curve

The linearity/calibration curve used in this validation process comprised of the following concentrations: 2.5, 5.0, 10.0, 25.0, 50 and 75.0 ng/ml.

The linear regression value found for monoamine analytes were as follows: dopamine (DA): r2 = 0.9989, norepinephrine (NE): r2 = 0.9987, serotonin (5-HT): r2 = 0.9986, and the

metabolites of these monoamines, 3,4-dihydroxyphenylacetic acid (DOPAC): r2 = 0.9979, 4-hydroxy-3-methoxyphenylacetic acid (HVA): r2 = 0.0.9989 and 5- hydroxyindole acetic acid (5-HIAA): r2 = 0.9985.

Lower limit of detection (LLOD)

The lower limit of detection was found to be 1.0 ng/ml.

Lower limit of quantification (LLOQ)

The lower limit of quantification was found to be 2.5 ng/ml, which was also the lowest concentration on the calibration curve.

A.6.2.2 Chemicals and reagents

Solution A:

Contents:

0.5 mM sodium metabisulphite
0.3 mM Na2EDTA
0.1 M perchloric acid (60% strong solution).

Preparation of Solution A:

(1) 0.09505 g of sodium metabisulphite and 0.111672 g Na2EDTA was weighed off and dissolve in 800 ml distilled water.
(2) 10.87 ml of perchloric acid was then added to the solution above and make up to 1000 ml.

Note: All standards were prepared with this solution.

A.6.3 Sample preparation of brain tissue

(1) Following dissection, brain tissue (frontal cortex) of each animal were placed individually into polypropylene tubes, marked and snap frozen with liquid nitrogen. The samples were stored at -86°C until the day of analyses.

(2) On the day of analysis, sample were weighed, thawed and 1 ml of solution A was added to each tube. The tissue in each tube was then ruptured by sonication (2 x 12 seconds, at an amplitude of 14 µ).

(3) The tubes were then left to stand on ice for a period of 20 minutes to complete perchlorate precipitation of protein and extraction of monoamines.

(4) Following this period, samples were centrifuges at 4°C in an ultra-centrifuge for 20 minutes at 16 000 rpm (24 000 g).

(5) The pH of the sample was adjusted to pH5 with the addition of 1drop (1 ml) of 10 M potassium acetate.

(6) An aliquot of 200 µl of the tissue extract was removed with a pipette and placed into another tube.

(7) 20 µl of the internal standard, isoprenaline, was added to the sample.

(8) The final sample was vortexed centrifuged and, thereafter, 10 µl was injected into the HPLC column.

(9) Results are expressed as ng/mg wet weight of tissue.

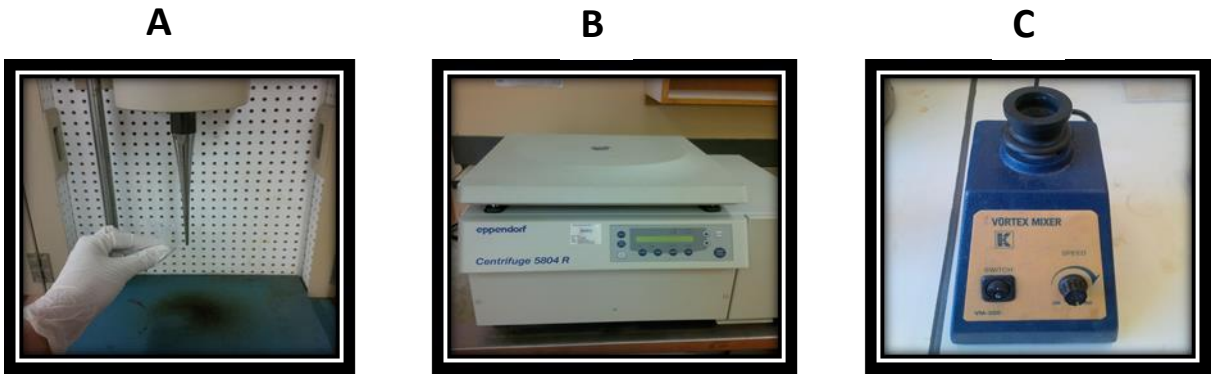

Figure A-7: Images of the sample preparation where (A) depicts the sonicater, (B) depicts the centrifuge and (C) depicts the vortex.

A.6.4 Statistical Analysis

When comparing only two data points, the Student's T test was used. However, for multiple comparisons of data, the two-way ANOVA was used, followed by the Tukey posthoc test if the two-way ANOVA indicated interaction between the main factors (i.e. rat line and drug treatment). In all cases GraphPad Prism® version 6 (GraphPad Software, San Diego California USA, www.graphpad.com) was used for statistical analysis and graphical presentations. A 5% confidence limit for error was taken as statistically significant ($p<0.05$). The Statistical Consultation Service of the North-West University was consulted before finalization of analyses.

Addendum B - Additional and Supplementary Results

This addendum depicts additional data that was not presented in Chapter 3 (Scientific Article), and serves as supporting material to the main study and still pertains to the study as a whole. The additional data showed slight differences between some treatment groups and was not regarded as being publishable in a scientific journal. One of the objectives of the current study was to draw a link between the observed behavioural changes in the animals and the monoamines levels and their metabolites in the frontal cortex. These were measured using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (Harvey *et al.,* 2006). The materials and methods of the neurochemistry analyses are fully described in Addendum A.

Monoamines determined in the study include dopamine (DA), norepinephrine (NE) and serotonin (5-HT) and the metabolites of these monoamines, 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 5- hydroxyindole acetic acid (5-HIAA). The methods used to determine these regional brain monoamines are described in Addendum A.

Chronic administration of METH has been documented to elevate levels of the extracellular monoamine neurotransmitters, such as DA, 5-HT, and NE (Bubenikova-Valesova *et al.,* 2009). Monoamine transporters (MATs) include the norepinephrine transporter (NET), the serotonin transporter (SERT), and the dopamine transporter (DAT) are located in the plasma membrane of the presynaptic nerve terminals from which the monoamine neurotransmitters are released (Haenisch & Bonisch, 2011). METH's ability to release DA rapidly in specific regions of the brain is responsible for the resulting intense euphoria. The activation of the reward system in the brain is associated with an increased activity of DA-containing pathways and not noradrenergic pathways. Although NE does not play a significant role in the reinforcing effects of psychostimulants, it has been reported that drugs that increase NE can share interoceptive effects with psychostimulants. Recent evidence also associates NE in stress- and drug-induced reinstatement of psychostimulant self-administration (Howell & Kimmel, 2008). The reinforcing and stimulant effects of psychostimulants are not directly dependent on 5-HT, however it has been demonstrated that pharmacological modulation of the serotonergic system may alter the behavioural and neurochemical effects of psychostimulants (Howell & Kimmel, 2008). The permissive hypothesis also states that manic and depressive episodes are characterised by a decreased central 5-HT function (Spencer, 1977). Serotonergic systems are responsible for inhibiting a range of other

neurotransmitter functions and consequently mood disorders result from the removal of this inhibition.

B.1 Results

Figure B-1 display the regional brain monoamine levels as measured in the frontal cortex of vehicle- and METH-treated FSL and FRL rats. DA, NE and 5-HT levels are presented in ng/mg of brain tissue. These figures display measurements at PnD35 (i.e. 24 hours after the last dose) and at PnD60 (i.e. long-lasting effects after washout).

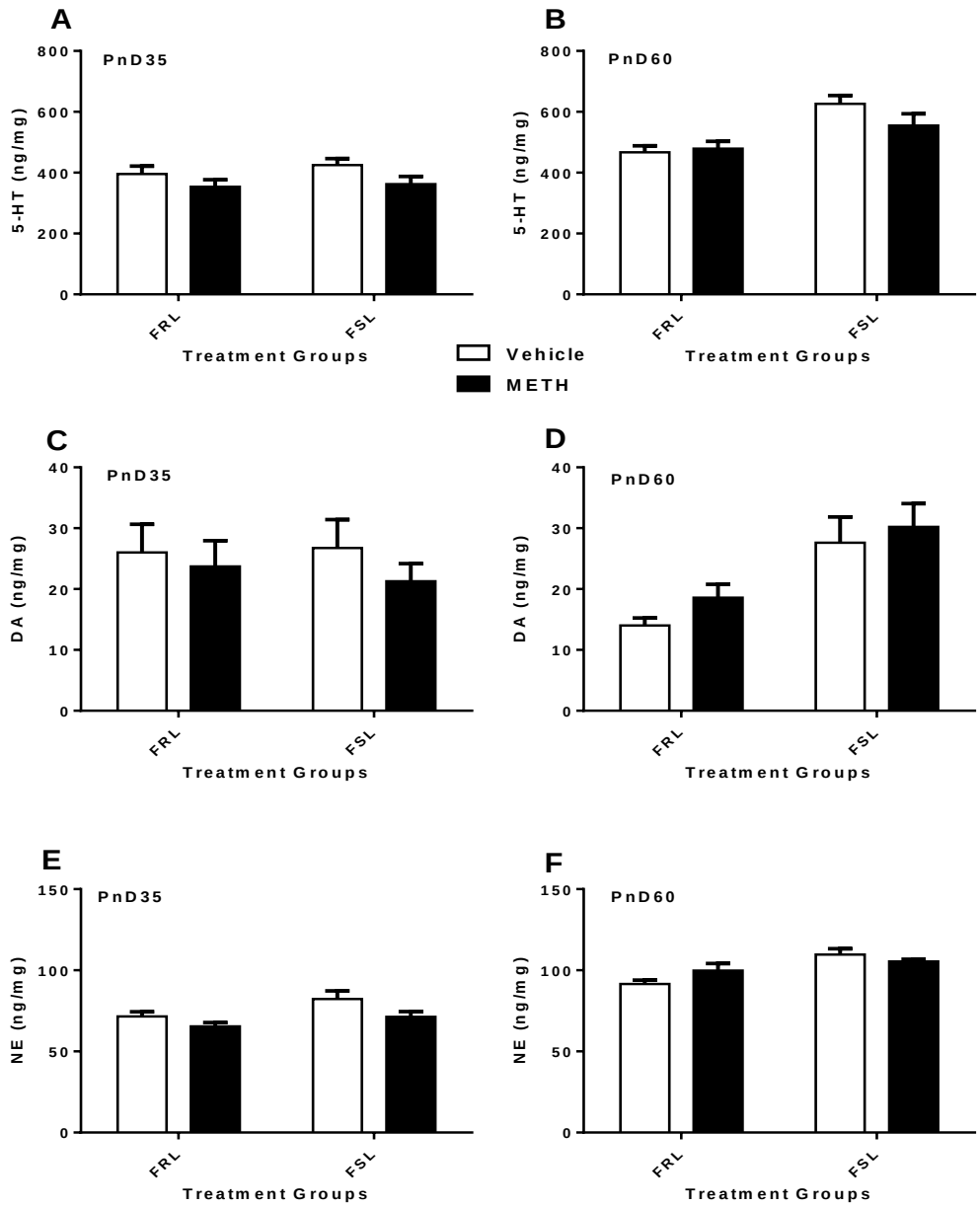

Figure B-1: The effect of vehicle versus chronic methamphetamine (METH) exposure in Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats on regional brain monoamine levels. 5-HT (A and B), DA (C and D) and NE (E and F) are presented at PnD35 (A, C and E) and PnD60 (B, D and F) respectively. Data points represent the mean ± S.E.M.

In Figure B-1A the two-way ANOVA of the data ($F(1, 51) = 0.1903$, $P = 0.6645$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding 5-HT levels on PnD35. The main factor effect of line of rat was also not significant ($F(1, 51) = 0.6289$, $P = 0.4314$), however drug treatment significantly decreased serotonin relative to controls ($F(1, 51) = 4.670$, $P = 0.0354$).

In Figure B-1B the two-way ANOVA of the data ($F(1, 44) = 1.986$, $P = 0.1658$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding 5-HT levels on PnD60. The main factor effect of line of rat was, however, statistically significant ($F(1, 44) = 15.96$, $P = 0.0002$), but the effect of drug treatment was not statistically significant ($F(1, 44) = 1.005$, $P = 0.3215$).

In Figure B-1C the two-way ANOVA of the data ($F(1, 49) = 0.1366$, $P = 0.7133$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding DA levels on PnD35. Both main factor effects of line of rat ($F(1, 49) = 0.03838$, $P = 0.8455$) and drug treatment ($F(1, 49) = 0.8239$, $P = 0.3685$) were not statistically significant.

In Figure B-1D the two-way ANOVA of the data ($F(1, 41) = 0.09009$, $P = 0.7656$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding DA levels on PnD60. The main factor effect of line of rat was, however, statistically significant ($F(1, 41) = 14.54$, $P = 0.0005$), although the effect of drug treatment was not statistically significant ($F(1, 41) = 1.179$, $P = 0.2840$).

In Figure B-1E the two-way ANOVA of the data ($F(1, 51) = 0.4082$, $P = 0.5257$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding NE levels on PnD35. However, both main factor effects of line of rat ($F(1, 51) = 4.560$, $P = 0.0376$) and drug treatment ($F(1, 51) = 4.890$, $P = 0.0315$) were statistically significant.

In Figure B-1F the two-way ANOVA of the data ($F(1, 43) = 3.926$, $P = 0.0540$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding NE levels on PnD60. The main factor effects of line of rat was statistically significant ($F(1, 43) = 13.95$, $P = 0.0005$), however the effect of drug treatment was not statistically significant $F(1, 43) = 0.4010$, $P = 0.5299$).

Figure B-2 display the regional brain monoamine metabolite levels as measured in the frontal cortex of vehicle- and METH-treated FSL and FRL rats. 5-HIAA, DOPAC and HVA levels presented in ng/mg of brain tissue. These figures display measurements at PnD35 (i.e. 24 hours after the last dose) and at PnD60 (i.e. long-lasting effects after washout).

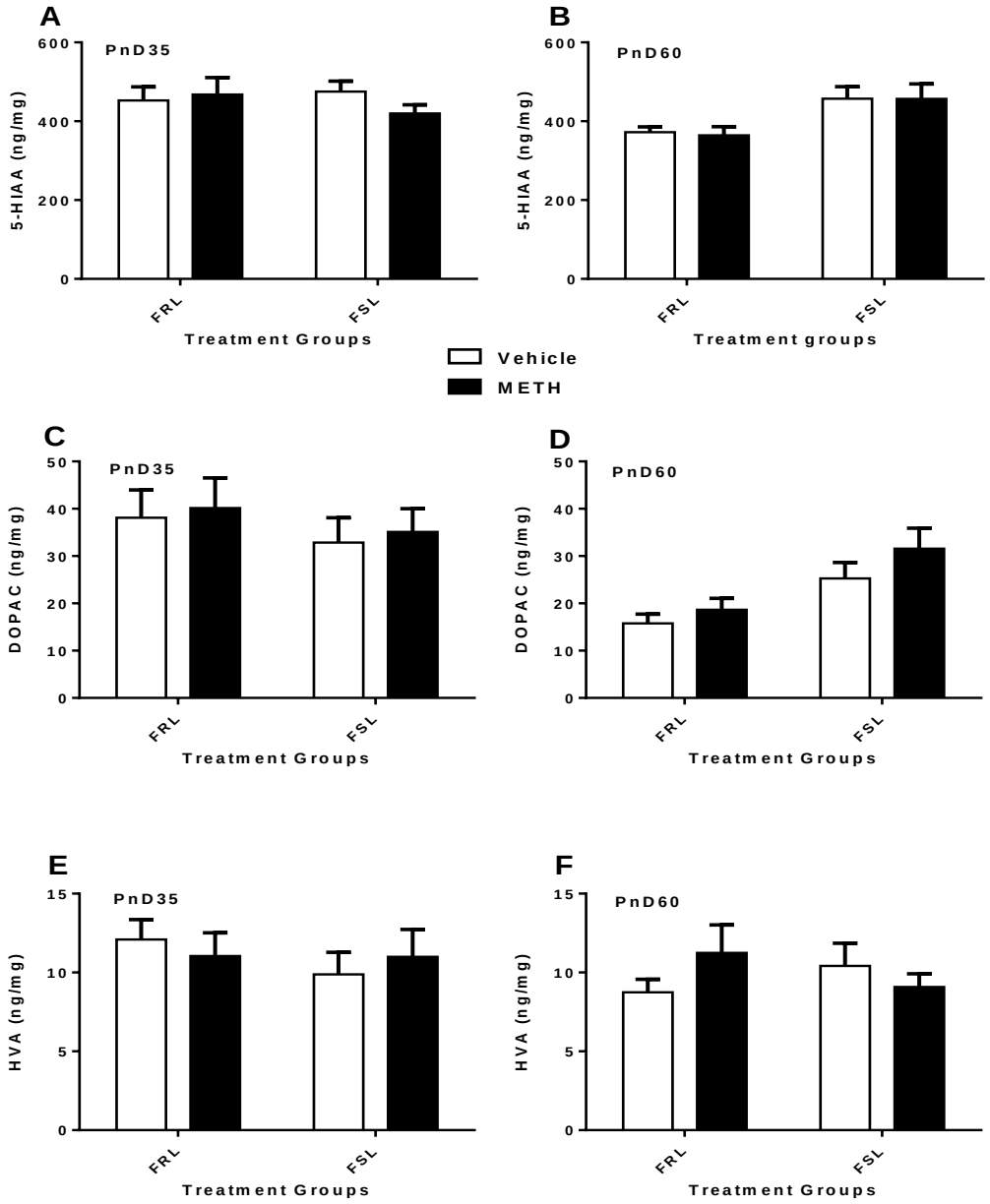

Figure B-2: The effect of vehicle versus chronic methamphetamine (METH) exposure in Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats on regional brain monoamine metabolite levels. 5-hydroxyindole acetic acid (5-HIAA) (A and B), 3,4-dihydroxyphenylacetic acid (DOPAC) (C and D) and 4-hydroxy-3-methoxyphenylacetic acid (HVA) (E and F) are presented at PnD35 (A, C and E) and PnD60 (B, D and F) respectively. Data points represent the mean ± S.E.M.

In Figure B-2A the two-way ANOVA of the data ($F(1, 50) = 1.223$, $P = 0.2740$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding 5-HIAA levels on PnD35. Both the main factor effect of line of rat ($F(1, 50) = 0.1617$, $P = 0.6893$) and drug treatment ($F(1, 50) = 0.4093$, $P = 0.5252$) was also not significantly.

In Figure B-2B the two-way ANOVA of the data ($F(1, 45) = 0.02093$, $P = 0.8856$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding 5-HIAA levels on PnD60. The main factor effect of line of rat was statistically significant ($F(1, 45) = 9.925$, $P = 0.0029$), however drug treatment was not statistically significant ($F(1, 45) = 0.02307$, $P = 0.8800$).

In Figure B-2C the two-way ANOVA of the data ($F(1, 51) = 0.0002906$, $P = 0.9865$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding DOPAC levels on PnD35. Both the main factor effect of line of rat $F(1, 51) = 0.8371$, $P = 0.3645$) and drug treatment ($F(1, 51) = 0.1453$, $P = 0.7046$) was not significant.

In Figure B-2D the two-way ANOVA of the data ($F(1, 41) = 0.2830$, $P = 0.5976$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding DOPAC levels on PnD60. The main factor effect of line of rat was statistically significant ($F(1, 41) = 11.98$, $P = 0.0013$) however drug treatment was not statistically significant ($F(1, 41) = 2.009$, $P = 0.1639$).

In Figure B-2E the two-way ANOVA of the data ($F(1, 51) = 0.5036$, $P = 0.4811$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding HVA levels on PnD35. Both the main factor effect of line of rat ($F(1, 51) = 0.5562$, $P = 0.4592$) and drug treatment ($F(1, 51) = 0.0003334$, $P = 0.9855$) was also not significant.

In Figure B-2F the two-way ANOVA of the data ($F(1, 44) = 2.254$, $P = 0.1404$) indicate that there was no statistically significant interaction between line of rat and drug treatment regarding HVA levels on PnD60. Both the main factor effect of line of rat ($F(1, 44) = 0.03460$, $P = 0.8533$) and drug treatment ($F(1, 44) = 0.2051$, $P = 0.6528$) was not statistically significant.

B.2 Discussion

METH significantly decreased frontal cortical 5-HT and NE levels relative to controls on PnD35 but not on PnD60. However, METH did not affect 5-HIAA or HVA levels significantly on PnD35 or on PnD60. A previous study conducted in our laboratory also found no differences in 5-HIAA levels as measured in the pre-frontal cortex (Strauss, 2012). Chronic use of METH causes depletion of monoamines stores and can thus explain why a decrease in 5-HT and NE was observed. However, data did not indicate a significant decrease in DA levels. A recent study investigated the effects of betulinic acid and METH on brain monoamines. Betulinic acid is a new promising anti-HIV agent and since many METH users are also HIV-positive, the effects of these two substances were tested in combination and it was documented that betulinic acid or METH alone did not decrease the levels of dopaminergic markers in the striatum, however co-administration of these agents decreased dopaminerigc makers in a betulinic acid dose-dependent manner (Killinger *et al.,* 2014). It is also documented that the adolescent brain, particularly the dopaminergic system is relatively resistant to METH neurotoxicity (Killinger *et al.,* 2014) while previous studies have found that adolescent rats are resistant to the neurotoxic effects of METH and have fewer DAT binding sites than adult rats. In addition, DA nerve endings in the nucleus accumbens are more resistant to the damaging effects of METH. METH-induced damage to DA terminals has been reported to be more severe in the dorsal striatum in comparison with the nucleus accumbens and prefrontal cortex, which is probably caused by the different densities of the DAT in these brain regions (Schwendt *et al.,* 2009). Thus the latter may explain why no significant changes were observed in DA levels (Thomas *et al.,* 2009). METH effects on 5-HT, however, are distributed throughout the frontal cortex (Schwendt *et al.,* 2009) and are in line with the current data presented.

Interestingly, an earlier study in SIR animals noted that while neither isolation rearing or METH separately or together (METH administered during puberty) altered corticostriatal 5-HT or NE levels, frontal cortical DA levels were significantly increased in SIR and METH treated animals separately, with a trend towards further elevated levels in combined METH plus SIR animals (Strauss *et al.,* 2014). Striatal DA was unaltered by all treatments. The authors concluded that the observed behavioural changes were more associated with altered frontal cortical but not striatal DA. Although the latter study is different in its design, it is perhaps relevant here as to the role of monoamines, especially DA, in the actions of METH.

According to the permissive hypothesis manic and depressive episodes are characterised by a decreased central 5-HT function (Spencer, 1977) where deficits in 5-HT can cause NE

levels to fall below normal ranges which then results in depression (Hilty *et al.,* 2006). METH causes the rapid release of 5-HT and NE into the synapse and may thus disrupt the balance between these neurotransmitters resulting in mood disorders such as depression. The METH-induced decrease in 5-HT could have causes the decrease in NE that was observed directly after METH treatment on PnD35. Thus, these observations are in line with the permissive hypothesis of depression and can be related to the depressive-like behavioural symptoms observed during behavioural testing in the current study.

Since METH is known to induce inflammation, the reduced 5-HT levels observed may also be the result of increased release of pro-inflammatory cytokines resulting in induction of IDO and the shunting of tryptophan metabolism toward the production of kynurenine and quinolinic acid rather than 5-HT, thus decreasing 5-HT levels (Soskin *et al.,* 2012). Increased quinolinic acid is toxic to neurons. Several studies implicate decreased serotonergic activity in major depression and acute trypotophan depletion studies support this theory.

The data also indicated that the main factor effect of line of rat was statistically significant regarding 5-HT levels on PnD60 where higher 5-HT levels were observed in the FSL rat controls (saline treated rats) relative to FRL rat controls. This finding is in line with the characteristics of the FSL rat model of depression where the FSL rat displays serotonergic abnormalities including elevated 5-HT content in the limbic regions (Yadid *et al.,* 2000). FSL rats displays increased 5-HT levels in the hippocampus, pre-frontal cortex as well as the nucleus accumbens (Yadid *et al.,* 2000).

Previous studies investigating the two-hit hypothesis using the maternal separation or SIR model have not been able to show greater abnormalities when combined with METH, while a bolstering effect was evident in FSL+METH-treated animals, as described here. A possible compensatory mechanism, or a ceiling effect, in response to METH may explain the lack of a bolstering effect observed following either maternal separation (Dimatelis *et al.,* 2014) or SIR (Strauss *et al.,* 2014). While maternal separation and isolation rearing are developmental animal models of depression (Fone & Porkess, 2008), the FSL rat is a genetic model. Their underlying inherent neurobiology are likely to differ, which on the one hand suggests that the FSL rat is more susceptible to multiple hits and thus better suited for studies investigating the dual hit hypothesis. On the other hand, it also indicates that genetic predisposition rather than early-life adversity amounts to a greater pre-existing risk factor for developing METH-associated sequelae later in life. Moreover, the current study, would suggest that this difference may lie in how these models affect monoamines. For example, SIR was found to induce significant deficits in frontal cortical DA and 5-HT but significantly elevating frontal

cortical NE as well as striatal DA and 5-HT (Moller *et al.,* 2013), which is markedly different to how monoamines were altered in FSL rats described here.

It appears that interactions between NE and DA may be vital in the behavioural pharmacology of psychostimulants. The NE system is generally neuroprotective and influences the vulnerability of nigrostriatal DA neurons (Winshenker *et al.,* 2008). The NE system possesses potent antioxidant properties and protects DA neurons from oxidative stress in vitro (Winshenker *et al.,* 2008). Thus the absence of endogenous NE increases the toxicity of METH on the DA system (Winshenker *et al.,* 2008). The increase in DA release via NE depletion following multiple doses of METH has been documented (Winshenker *et al.,* 2008). A decrease in NE was observed on PnD35, however the consequent significant increase in DA was not seen in the current study. In addition HVA levels were not significantly altered by METH. The data indicated, however, that the main factor effects of line of rat was statistically significant regarding NE levels on PnD60 where the saline treated FSL rats displayed increased NE levels compared the saline treated FRL rats. This has been documented previously in the FSL rat where increased NE was measured in the nucleus accumbens, pre-frontal cortex and the hippocampus (Yadid *et al.,* 2000).

It has been hypothesised that behavioural sensitisation results from an increase in DA release in the mesolimbic and nigrostriatal dopaminergic terminals, after the administration of psychostimulants (Bubenikova-Valesova *et al.,* 2009). Previous studies indicated that acute administration of METH releases DA, but decreases DOPAC and HVA (Bubenikova-Valesova *et al.,* 2009). In addition, METH inhibits DA reuptake and monoamine oxidase (MAO), which catabolises DA into DOPAC. Both mechanisms decrease DOPAC levels after the administration of METH (Bubenikova-Valesova *et al.,* 2009). The current data indicated a slight increase in DA levels on PnD60, however it was not statistically significant. METH treatment did not indicate any significant changes in DA levels. Disruption in dopaminergic function during pre-adolescence can still influence behaviour that persists later in life. Due to the fact that METH also attenuates monoamine metabolism, by inhibiting MAO (Cruickshank & Dyer, 2009), the metabolism of DA to DOPAC should be inhibited with an eventual decrease in DOPAC, however, this effect was not indicated in our data. The data indicated, however, that the main factor effects of line of rat was statistically significant regarding DA levels on PnD60 where the saline treated FSL rats displayed increased DA levels compared the saline treated FRL rats. So while we observed no significant differences in DA before or after withdrawal from METH, 5-HT and NE alterations may have caused the noteworthy behavioural deficits as presented in Chapter 3.

Neurochemical analyses did provide some insight to the neurochemical mechanism of how METH causes or worsens depression, some alterations in monoamines were observed and can be related to behavioural changes seen. The behavioural data and linked neurochemical changes are discussed in detail in Chapter 4 (Summary § 4.1).

Addendum C - Instructions to the author

This addendum includes the "instructions to the author" that serve to indicate the guidelines set out by the specific journal to with the article (Chapter 3) will be submitted.

Instructions to the author that pertains to the International Journal of Developmental Neuroscience can be found at [Online]:
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Addendum D - Congress contribution and Young Scientist award

This addendum describes the study results that were presented as a poster at the 17th World Congress of Basic and Clinical Pharmacology in July 2014 (WCP2014) in Cape Town, South Africa. During the closing ceremony of the WCP2014, I received the South African Society for Basic and Clinical Pharmacology (SASBCP) 1st Prize award for my academic poster in the basic pharmacology category. The poster was titled:

The long-lasting effects of early-life administration of methamphetamine on depressive-like behaviour in stress-sensitive rats

Mouton, M., Swart, C., Harvey B.H., Brink, C.B.
Unit for Drug Research and Development, Faculty of Health Sciences, North-West University, Potchefstroom, 2520, South Africa.
Correspondence: Tiaan.Brink@nwu.ac.za

Introduction

The psychostimulant methamphetamine (METH) is a drug of abuse. Due to easy access to its precursors, relative simple synthesis and low cost, it has reached epidemic proportions globally (Cruickshank & Dyer, 2009) particularly amongst the youth. METH use is associated with significant health risks (Dipiro *et al.,* 2011) including psychosocial dysfunction and psychiatric disease such as major depression (Sutcliffe *et al*., 2009). Exposure during early-life development may result in neurobehavioural deficits later in life. This study investigated the effect of early-life chronic METH exposure on depressive-like behaviour in stress sensitive Flinders Sensitive Line (FSL) versus control Flinders Resistant Line (FRL) rats, both directly following METH exposure and after withdrawal later in early adulthood.

Study Aims and Objectives

To investigate the effect of early-life chronic administration of METH to stress-sensitive and control rats on depressive-like behaviour, directly after the injection period on post-natal day 35 (PnD35), as well as after withdrawal in early adulthood on PnD60.

Research Methodology

Animals

The current study employed Flinders sensitive line (FSL) and Flinders resistant line (FRL) rats. FSL rats represent a widely described and validated genetic animal model of depression (Overstreet & Wegener, 2013) with face, predictive and construct validity (Overstreet *et al.,* 2005), with FRL rats representing normal controls. Animals were housed under controlled conditions: temperature 22 ± 1°C, humidity 50%, 12:12-h light/dark cycle, food and water ad libitum. All experiments conformed to the guidelines of the National Institutes of Health for the care and use of laboratory animals, and were approved in accordance with the regulations set by the Research Ethics Committee-Animals of the North-West University (ethics approval no. NWU-000105-11-S5).

Treatment Regimen

FSL and FRL rats (n = 16 per treatment group) were administered METH or vehicle from PnD19 to PnD34, i.e. 16 days during pre-adolescence. Subcutaneous administrations were performed twice daily at 09:00 and 15:00, with incremental dose increases of METH (0.2 - 6.0 mg/kg, increasing with 0.2 mg/kg per administration). Thereafter rats were either directly subjected to the forced swim test (FST) on PnD35, or were housed normally (withdrawal phase) until PnD60, and then subjected to the FST as described below.

Behavioural Testing

Behavioural testing commenced after the start of the dark cycle, between 18:00 and 24:00. Depressive-like behaviour were assessed using the FST (Abildgaard *et al.,* 2011), where rats were placed in an inescapable cylinder of water and escape-oriented versus immobile behaviour (just sufficient movement to keep the head above water) (Cryan *et al.,* 2002) measured during a 5 minute swim trial. FSL rats display inherently enhanced immobility and no pre-conditioning swim trial is required.

Results

FSL rats displayed depressive-like behaviour (enhanced immobility) relative to FRL rats. Chronic METH exerts antidepressant-like effects prior to withdrawal on PnD35 (adolescence) in both FRL and FSL rats. Following 25 days withdrawal, METH exerts depressive-like effects (enhanced immobility) on PnD60 (early adulthood) in FRL rats, and augmented the enhanced depressive-like behaviour in genetically predisposing FSL rats.

Conclusion

Pre-adolescent administration of METH for 16 days induces mild antidepressant-like behaviour before withdrawal (PnD35, adolescence), putatively associated with its psychostimulant effects. However, after 25 days withdrawal (PnD60, early adulthood), this was reversed and the depressogenic effects were observed in FRL and FSL rats. It may be deduced that pre-adolescent exposure of rats to METH results in long-lasting depressogenic effects, putatively via altered neurodevelopment (for further investigation). This long-lasting effect also seems to be additive to the congenital depressive-like phenotype in FSL rats, suggesting a role for genetic susceptibility. This observation would be in line with a two-hit hypothesis of depression (Nabeshima & Kim, 2013), suggesting that the manifestation of depression will result when genetic predisposition is followed by an environmental stressor (i.e. METH) later in life. The data suggest as working hypothesis that individuals that already have a predisposition to depression may be more susceptible to developing depression when abusing METH.

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