Effects of early-life administration of methamphetamine on the depressive-like behaviour later in life in stress-sensitive and control rats

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Dissertation submitted in partial fulfillment of the requirements for the degree *Magister Scientiae* in Pharmacology at the Potchefstroom Campus of the North-West University

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November 2013

“Pain is inevitable. Suffering is optional.”

-Haruki Murakami

Spreuke 3:5-6 – Vertrou op die Here met jou hele hart en steun nie op jou eie insig nie. Ken hom in al jou weë, dan sal Hy jou paaie gelykmaak.

Abstract

Methamphetamine (MA) is a well-known, easily accessible and powerful psychostimulant, and its abuse has become a global problem. MA abuse affects millions of people worldwide and places an enormous burden on public healthcare resources. Documented consequences of MA abuse include cardiotoxic, neurotoxic and teratogenic effects, as well as long-term consequences of chronic abuse including affective disorders such as schizophrenia and major depressive disorder (MDD). MDD is a highly prevalent mood disorder in both adults and children, documented to contribute to approximately 850 000 suicides annually. This disorder is projected to become the 2nd leading disease of global burden by 2020, preceded only by ischemic heart disease. Depressive-like behaviour is documented as a symptom of chronic MA abuse and particularly during extensive MA withdrawal. Also, MA abuse during pregnancy is documented to cause neurodevelopmental changes that persist into later life. However, current understanding thereof is limited and warrants further investigation of the effects of early-life exposure to MA on outcome in adulthood, particularly in terms of mood disorders.

The aim of the current study was to determine the effect of chronic exposure to MA on the depressive-like behaviour later in life in stress-sensitive (Flinders Sensitive Line) and control (Flinders Resistant Line) rats. Rats were exposed during one of the following natal day (ND) age groups: prenatal (ND-13 to ND+02), postnatal (ND+03 to ND+18), prepuberty (ND+19 to ND+34) or puberty (ND+35 to ND+50). These age groups represent different stages in neurodevelopment, as also seen in humans. For prenatal exposure, pregnant dams received 5 mg/kg daily subcutaneously (s.c.), and pups from postnatal, prepuberty and puberty age groups received an escalating dose regimen to simulate "binge-dosing" commonly seen in humans abusing MA. After MA exposure, rats were housed normally until behavioural testing on postnatal day 60 (ND+60), which included the novel object recognition test (NOR), open field test (OFT) and forced swim test (FST), measuring cognitive function, locomotor activity and depressive-like behaviour respectively.

The FST data showed increased immobility behaviour of saline-treated FSL rats relative to that of FRL rats, in line with previous data validating FSL rats as a genetic rodent model of depression. Practically significant MA-induced increases in immobility behaviour were observed in all FSL and FRL treatment groups in the FST, reaching statistical significance in prenatally treated FRL rats, and in postnatally, prepuberty and puberty treated FSL rats. The data suggest that early-life MA exposure may alter neurodevelopment to predispose the rats to display depressive-like behaviour in early adulthood, and suggests that this detrimental effect of

MA may be more expressed in stress-sensitive rats. Furthermore, all FSL groups plus prenatally and puberty treated FRL rats revealed practically and statistically significant decreases in swimming behaviour in the FST, whereas decreases in swimming behaviour in prepuberty treated FRL rats were practically significant but did not reach statistical significance. These data suggest that MA-induced depressive-like behaviour in FSL rats may be related to impaired serotonergic neurotransmission, and that this appears to be more robust in FSL rats. Climbing behaviour in the FST was generally not altered by early-life MA exposure, with a notable exception being a practically and statistically significant increase in puberty treated FRL rats. These data suggest that in general early-life MA exposure does not affect noradrenergic neurotransmission in early adulthood, except when normal rats were treated at puberty. The reason for the latter observation is not clear. The data from the NOR test revealed no discernible trends of MA-induced effects on memory and cognition, except for a small albeit practically significant increase in exploration time in prepuberty treated FRL rats and a practically and statistically significant decrease in exploration time in puberty-treated FRL rats. Lastly, locomotor activity in the OFT was mostly unaffected by MA treatments, except for practically significant decreases in locomotor activity in postnatally-and prepuberty-treated FRL rats and practically and statistically significant decreases in locomotor activity of prepuberty treated FSL rats. Altered locomotor activity is therefore not expected to explain any of the immobility results of the FST.

In final conclusion, the study confirms that early-life MA exposure results in a depressogenic effect later in life in stress-sensitive (FSL) and control (FRL) rats, but appears to be more robust in stress-sensitive animals. Furthermore the data suggest that long-lasting MA-induced depressogenic effects may relate to impaired serotonergic neurotransmission.

Keywords: methamphetamine, depression, neurotoxicity, teratogenic, Flinders Sensitive Line rat, depressive-like behaviour

Opsomming

Metamfetamien (MA) is ’n dwelmmiddel wat bekend is weens die misbruik daarvan – en berug vanweë die maklike verkrygbaarheid en kragtige psigostimulerende eienskappe daarvan. Die misbruik van MA is tans ’n wêreldwye probleem wat enorme druk plaas op hulpbronne in die publieke gesondheidsorgsektor. Genoteerde gevolge van MA-misbruik sluit kardiotoksiese, neurotoksiese en teratogeniese effekte in. Langtermyngevolge van chroniese MA-misbruik sluit die moontlike ontwikkeling van affektiewe versteurings in, waaronder skisofrenie en major depressiewe versteuring (MDV). Major depressiewe versteuring (MDV) kom algemeen voor in beide kinders en volwassenes en dra jaarliks by tot sowat 850 000 selfmoorde. Daar word beraam dat hierdie steurnis teen 2020 die tweede voorste afwyking van globale omvang sal wees; slegs oortref deur iskemiese hartsiekte. Depressief-agtige gedrag is aangeteken as ’n simptoom van kroniese MA-misbruik, in die besonder gedurende uitgebreide MA-onttrekking. Verder kan MA-misbruik tydens swangerskap afwykings in neuro-ontwikkeling veroorsaak wat voortduur tot in latere lewe. Die huidige wetenskaplike begrip daarvan is egter beperk en onderskraag derhalwe verdere ondersoek na die blywende uitwerking wat MA-blootstelling tydens vroeë lewensfases het op volwasse gesondheid, in die besonder betreffende gemoedsversteurings.

Hierdie studie het dit ten doel om die effek van kroniese blootstelling aan ’n neurotoksiese MA-dosis op die ontwikkeling van depressief-agtige gedrag na puberteit te bepaal in stres-sensitiewe (Flinders Sensitiewe Lyn, oftewel FSL) en kontrole- (Flinders Weerstandige Lyn, oftewel FWL) rotte. Die rotte is blootgestel gedurende een van die volgende ouderdomsfases (gemeet in natale dae: ND): prenataal (ND-13 tot ND+02), postnataal (ND+03 tot ND+18), pre-puberteit (ND+19 tot ND+34) of puberteit (ND+35 tot ND+50). Elkeen van hierdie ouderdomsfases verteenwoordig ’n spesifieke stadium in die ontwikkeling van menslike neurotransmittorsisteme. Gedurende prenatale blootstelling het die swanger rotte elk ’n 5 mg/kg daaglikse subkutaneuse (s.c.) MA inspuiting ontvang. ’n Stelselmatig-toenemende-dosis-toedieningstrategie is gebruik vir die postnatale, prepuberteit- en puberteitgroepe om die fuifgebruik (sogenaamde *binge-dosing*) van MA-verslaafde mense na te boots. Na MA-blootstelling is die rotte onder standaard omstandighede aangehou tot en met die gedragstoetse, wat plaasgevind het op postnatale dag 60 (ND+60). Die gedragstoetse sluit die nuwe–voorwerp-herkenningstoets, geforseerde swemtoets (GST) en lokomotoraktiwiteitstoets in, en meet onderskeidelik kognitiewe funksionering, depressief-agtige gedrag en lokomotoraktiwiteit.

Immobiliteitsgedrag, soos gemeet in die GST, was verhoog in alle FSL-rotte in vergelyking met FWL-rotte wat nie aan MA blootgestel is nie. Hierdie gedrag stem ooreen met data van vorige studies waarin die FSL-rot as genetiese diere-model van MDV bevestig is. Prakties-beduidende toenames in MA-geïnduseerde immobiliteitsgedrag is waargeneem in beide FSL- en FWL-rotte tydens die GST, waarvan die prenatale FWL groep en postnatale, prepuberteit- en puberteit-FSL-groepe statisties beduidende verskille getoon het. Hierdie bevindings suggereer dat vroeë-lewe MA-blootstelling kan lei tot wysigings aan neuro-ontwikkeling, wat gevolglik ’n groter vatbaarheid vir ontwikkeling van depressief-agtige gedrag in latere lewensfases veroorsaak. Uit die datastel blyk dit dat die nadelige gevolge van MA-blootstelling meer algemeen voorkom in stres-sensitiewe rotte. Daarbenewens het alle FSL groepe asook prenatale en puberteit FWL-rotte prakties- en statisties-beduidende afnames in swemgedrag getoon, soos gemeet aan die GST. Afnames in swemgedrag in die prepuberteit-behandelde FWL-rotte is prakties-beduidend, maar nie statisties-beduidend nie. Hierdie blyk aan te toon dat MA-geïnduseerde depressief-agtige gedrag in FWL- en FSL-rotte moontlik verwant kan wees aan belemmerde serotonergiese neuro-oordrag en dat dit meer algemeen sou voorkom in FSL-rotte. Klimgedrag in die GST was oor die algemeen onveranderd deur die vroeë-lewe MA-blootstelling, behalwe vir ’n prakties- en statisties-beduidende toename in puberteit-behandelde FWL-rotte. Die data toon dus aan dat MA-blootstelling oor die algemeen nie noradrenergiese neuro-oordrag verander in latere lewe nie, met die uitsondering van normale rotte wat behandel is tydens puberteit. Die rede hiervoor is nie duidelik nie. Data vanuit die nuwe-voorwerp-herkenningstoets toon geen merkbare neigings tot veranderde geheue of kognitiewe funksie as gevolg van MA-blootstelling nie. ’n Prakties-beduidende toename in verkenningstyd in prepuberteit-behandelde FWL-rotte en ’n prakties- en statisties-beduidende afname in verkenningstyd in puberteit-behandelde FWL-rotte is wel opgemerk. Laastens, lokomotoraktiwiteit, soos gemeet in die lokomotoraktiwiteitstoets, het meestal onveranderd gebly ná MA-blootstelling. Prakties-beduidende afnames in lokomotoraktiwiteit in postnatale en prepuberteit-behandelde FWL-rotte en prakties- en statisties-beduidende afnames in lokomotoriese aktiwiteit van prepuberteit-behandelde FSL-rotte is wel waargeneem. Veranderings in lokomotoriese aktiwiteit kan dus nie die immobiliteitsgedrag in die GST nie verklaar nie.

Die finale gevolgtrekking bevestig die depressogeniese effek van vroeë-lewe MA-blootstelling op gedrag in latere lewensfases in beide stres-sensitiewe (FSL) en kontrole (FWL) rotte. Die effekte van MA-blootstelling blyk wel meer algemeen voor te kom in stres-sensitiewe rotte. Daarbenewens suggereer die data dat langtermyn MA-geïnduseerde depressogeniese effekte moontlik verwant kan wees aan belemmerde serotonergiese neuro-oordrag.

Sleutelwoorde: metamfetamien, major depressie, neurotoksisiteit, teratogenies, Flinders Sensitiewe Lyn rot, depressiewe gedrag

Acknowledgements

First and foremost, glory to God our heavenly Father who created such a complex world that mankind will take all eternity to discover it. He blessed me with the strength, determination and patience and carried me during this experience.

My sincere thanks to my supervisor Prof. Christiaan Brink for the guidance and advice that helped me shape the literature, data and my thoughts into a dissertation and an achievement I can be proud of.

Many thanks to my co-supervisor Prof. Brian Harvey for the input and suggestions during this time which helped form the product of this experience. A special thanks to Prof. Linda Brand for all the consideration and attendance to new students and personnel.

To my family, thank you for your love, support and advice, not only in this endeavour but also during the years that led up to this day. Bernarda, Isobel, Marianne, Annemarie and my late sister Susan, thank you with all my heart.

To my friends, colleagues and fellow students, thank you for making these past years interesting, informative, exciting and memorable. Stephanie, Lindi, Deon, Laetitia, Marissa, Riaan, Sarel, Stephan, Renier, Nico, Moné, Dewet, Madeleine, Francois and to the Pharmacology staff and lecturers from who I learned so much.

To the Vivarium personnel and the team in Edenvale, thank you for your advice, help and support: Cor Bester, Antoinette Fick and Ingrid Linnekugel.

My thanks to everyone involved; your contributions have been immeasurable.

The financial assistance if the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Congress proceedings

Excerpts from the current study have been presented at the annual congress of pharmacology and family medicine (ACPFM 2012) as follows:

Effects of early-life exposure of methamphetamine on depressive-like behaviour in stress-sensitive rats

Swart, C., Harvey, B.H., Stein, D.J. & Brink, C.B. 2012

The excerpt was presented at the podium for the young scientist competition during the 46th annual congress of the South African Society for Basic and Clinical Pharmacology (SASBCP) in association with the department of Family Medicine (UP) and the Toxicology Society of South Africa (TOXSA) in Pretoria, South Africa (30 Sept-2 Oct 2012).

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A	
ADHD	attention deficit-hyperactivity disorder
AIDS	acquired immunodeficiency syndrome
AMPT	α-methyl-*p*-tyrosine
ATS	amphetamine-type substance
B	
BDNF	brain-derived neurotrophic factor
C	
CMS	chronic mild stress
CNS	central nervous system
COMT	catechol-O-methyl transferase
CRF	corticosterone-releasing factor
CRH	corticotrophin-releasing hormone
D	
D_1	dopamine receptor-type 1
D_2	dopamine receptor-type 2
DA	dopamine
DAT	dopamine transporter
DBH	dopamine-β-hydroxylase
DFP	diisofluorophosphates
DOPAC	3,4-dihydroxyphenylacetic acid
DSM-IV	diagnostic and statistical manual of mental disorders IV
DTG	di-O-tolylguanide
E	
ED	embryonic days
F	
FDA	food and drug administration
FRL	flinders resistant line
FSL	flinders sensitive line
FST	forced swim test
G	
GI	gastro-intestinal

GM	gestational months
	H
HIV	human immunodeficiency virus
5HT	5-hydroxytryptophan (serotonin)
HPA	hypothalamus-pituitary-adrenal
	L
L-DOPA	l-dihidroxyphenylalanine
	M
M_1	muscarine receptor-type 1
M_2	muscarine receptor-type 2
MA	methamphetamine
MAO	monoamine oxidase
MAO-A	monoamine oxidase type A
MAO-B	monoamine oxidase type B
MAOI	monoamine oxidase inhibitor
MDD	major depressive disorder
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
	N
NAC	n-acetyl cysteine
nAChR	nicotinic acetylcholine receptor
NARI	noradrenaline reuptake inhibitor
ND	natal day
NDRI	norepinephrine dopamine reuptake inhibitor
NE	norepinephrine
NET	norepinephrine transporter
NMDA	n-methyl-d-aspartate
NO	nitric oxide
NOR	novel object recognition
NOS	nitric oxide synthase
	O
OFT	open field test
	P
PND	postnatal days
PNW	postnatal weeks

R	
REM	rapid eye movement
RNS	reactive nitrogen species
ROS	reactive oxygen species
S	
$5HT_{1A}$	serotonin receptor-type 1A
$5HT_2$	serotonin receptor-type 2
σ_1	sigma receptor-type 1
σ_2	sigma receptor-type 2
SA	South Africa
SEM	standard error of the mean
SERT	serotonin transporter
SNRI	serotonin and noradrenaline reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
SwLo	swim-low active
T	
TAAR-1	trace amine-associated receptor type 1
TCA	tricyclic antidepressant
TH	tyrosine hydroxylase
TrH	tryptophan hydroxylase
U	
UNODC	United Nations Office on Drugs and Crime
USA	United States of America
V	
VMAT-2	vesicular monoamine transporter-2
W	
WHO	World Health Organisation

1 Chapter 1 Introduction

This Chapter provides the reader with an overview of the problem statement, study objectives, study layout and expected outcomes. It also describes the dissertation approach and layout.

1.1 Dissertation layout

The dissertation is presented in traditional format, consisting of five Chapters, subdivided as follows:

- Introduction to the dissertation (Chapter 1)
- Literature review (Chapter 2)
- Materials and methods (Chapter 3)
- Results and discussion (Chapter 4)
- Summary, conclusion and recommendations (Chapter 5)

1.2 Problem statement

Methamphetamine (MA) is a highly addictive and easily accessible psychostimulant that has become a widespread drug of abuse. Over the past two decades, MA use has become a growing problem in a number of regions, including the United States of America, Australia, Asia and South Africa (Plüddemann *et al.,* 2010, McKetin *et al.,* 2006). Increased treatment admissions in psychiatric hospitals related to MA abuse causes concern regarding the proliferated abuse of this drug (Plüddemann *et al.,* 2008). MA floods the neuronal synapses with dopamine (DA), serotonin (5HT) and norepinephrine (NE) by reversing the functions of the transport mechanisms for the respective monoamines. This results in a variety of peripheral and psychiatric symptoms. Psychiatric effects of MA use range from increased cognitive function and euphoria to anxiety, paranoia and psychotic episodes. The detrimental effects of MA abuse appear not only as long-lasting psychiatric symptoms but also as physical harm. The cardiotoxic properties of MA are well documented and can be exacerbated by concomitant use/abuse of other drugs (Darke *et al.*, 2008). The severe physical and psychological untoward effects of MA abuse commonly become irreversible with chronic use.

MA toxicity are strongly associated with glial cell activation, auto-oxidation of DA and the production of free radicals, all of which create an environment that indices oxidative stress. DA auto-oxidation and the resulting oxidative metabolites have the potential to cause long lasting damage to surrounding neural structures. Both preclinical (rat) and clinical studies on MA neurotoxicity suggest long-lasting structural and metabolic changes in the cortex and striatum

(Chang *et al.*, 2007; Howard *et al*., 2011; Iwazaki *et al*., 2006; Sekine *et al*., 2008), explaining the behavioural changes observed in chronic MA-use.

MA abuse has long been associated with psychiatric disorders such as major depressive disorder (MDD) and schizophrenia. In fact, there is a high and well-documented correlation between the development of mood disorders and a co-existence of genetic predisposition and environmental stressors, especially when these stressors occur in early-life developmental stages. MDD is the cause of about 850 000 deaths by suicide each year and is projected by the world health organisation to become the 2nd leading disease of global burden second to ischemic heart disease by 2020 (Andersen, 2003; Holden, 2000; WHO, 2012). Although characteristics of MDD may vary with each individual, they normally include several of the following symptoms: changes in eating or sleeping patterns, feelings of guilt and despair, loss of interest in activities, difficulty concentrating and low energy levels. Treatment options for MDD are extensive, yet one third of patients experience a relapse after treatment. The manifestation of MDD-associated symptoms is the most common following MA withdrawal and can also be present with active use. Withdrawal-induced depression can last up to 2 years in severe cases, and may lead to suicide (Zweben *et al.*, 2004; Meredith *et al.*, 2005).

MA abuse increased dramatically in many countries and has recently become a problem in South Africa. Increases in MA-related treatment admissions to psychiatric hospitals and documented MA use in schools is a cause of concern regarding the health of South African youth, particularly since MA use is associated with increased risk for a deterioration of mental and social health (Plüddeman *et al.*, 2010). Only a handful of studies describe the current situation of MA abuse in South Africa, most of which focus on the Western Cape Province, known for its high prevalence of MA abuse (Parry *et al.*, 2011; Plüddeman *et al.*, 2008; Plüddeman *et al.*, 2010; Vos *et al.*, 2010; Weich & Pienaar, 2009). Considering the widespread abuse of this destructive drug, far too few studies investigate the effect of MA on foetal and early-life neurodevelopment. Investigations into the effects of MA on neurodevelopment in animals have thrown some light on the consequences of prenatal MA-exposure. Therefore the current preclinical study proposes to supplement this gap by investigating the effects of early-life MA exposure on late-life behaviour.

MA-exposure during early-life development plays a crucial role in the outcome of late-life behaviour and wellbeing. The maturation of the neurotransmitter systems determines the stage of neurodevelopment when exposure to MA will cause the most damage. The serotonergic system is the first to mature in both the rat and the human, therefore the noradrenergic and

dopaminergic systems are still developing during childhood and adolescent phases and can be negatively influenced by MA exposure (Murrin *et al.*, 2007). MA can therefore affect an individual directly by abuse of the drug or indirectly via *in utero* exposure.

Prenatal exposure to centrally active substances such as alcohol is documented to have lasting consequences on the foetus, such as foetal alcohol syndrome, among many others. Lipophilic drugs have the ability to penetrate the placenta and alter the neural development to an extent that it is detected in late-life behavioural deficits. The current study therefore aims to determine the long-term effects of early-life MA exposure on the behaviour of stress-sensitive and control rats.

1.3 Project hypothesis and objectives

1.3.1 Aims and objectives

The current study aims to investigate in a rodent model of depression the effects of early-life exposure to MA on the manifestation of depressive-like behaviour in early adulthood, with specific reference to whether:

- exposure to MA at different stages of development provide different behavioural outcomes
- the effect of MA has is exacerbated by genetic susceptibility (i.e. stress-sensitivity)

1.3.2 Working hypotheses and expected outcomes

We postulate that early-life chronic administration of MA will:

- affect neurodevelopment such that depressive-like behaviour, locomotor activity and memory acquisition will be negatively affected during early adulthood
- affect depressive-like behaviour, locomotor activity and memory acquisition differently depending on the early-life age of administration of MA
- result in more pronounced behavioural deficits in rats that present with genetic predisposition for stress-sensitivity than in control rats.

1.4 Study layout

The current study was designed as for behavioural analysis following chronic MA-exposure during four different age groups (see below). The dosages that were administrated to each respective treatment group are based on previous studies and their outcomes concerning mortalities and neurotoxicity (see Table 3-2 in Chapter 3). Age was measured in natal days

(ND) and indicated with a positive or negative sign whether the age is prenatal or postnatal (e.g. ND-21 is the first day of gestation). Four age-appropriate treatment groups were selected and termed prenatal (ND-13 to ND+2), postnatal (ND+3 to ND+18), prepuberty (ND+19 to ND+34) and puberty (ND+35 to ND+50), during which they were treated with either MA or a saline control. Thereafter rats were housed under normal conditions and behaviour assessed at the age of ND+60. Behavioural tests included the forced swim test (FST) to evaluate depressive-like behaviour, the open field test (OFT) to assess locomotor activity and the novel object recognition (NOR) test to evaluate acquisition memory/cognition. Figure 1-1 gives a schematic representation diagram of the study layout, which is discussed in more detail in Chapter 3.

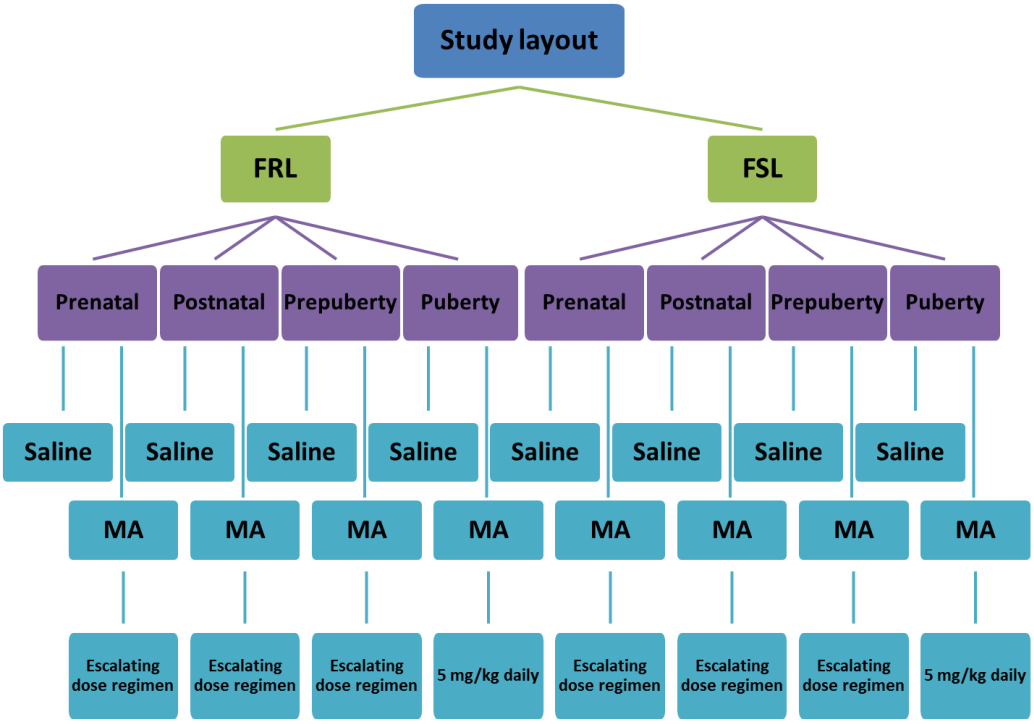

Figure 1-1: It can be seen that all studies were performed in both FRL and FSL rats, and that for each rat line the four age groups were treated. Furthermore, each age group received either vehicle or MA at the indicated dose regimen.

2 Chapter 2 Literature review

Drug abuse and the consequent disturbance of mental health remains a difficult challenge facing health care professionals today. The central stimulant MA presents a high risk for such abuse and subsequent detrimental effects on health. Decreased cognitive function, developmental retardation and mood disorders are well documented as a consequence of neurodegeneration caused by drug abuse. Defining the key factors that influence the outcome of MA exposure and abuse will save lives and resources, and will further our knowledge about the neurobiological basis of MA-induced neurodegeneration.

MDD is a well-described and serious psychiatric disorder with a high prevalence in both adults and children. Being a significant global burden, a great deal of resources has been devoted to a better understanding of the disorder, including its neurobiological basis and treatment. Due to the limitations and ethical difficulties associated with human studies, as well as the limitations of data provided by *in vitro* studies, many research approaches have begun to focus on the development of animal models of the human condition. For example, selective breeding of rodent strains has in recent years produced animal models that closely resembles certain features of MDD in humans, such as the Flinders Sensitive Line (FSL) rats (Overstreet *et al.*, 2005), stress-sensitive hypertensive rats (Boldyrev *et al.*, 1990), the fawn-hooded rat (Rezvani *et al.*, 2002) and BALB/c mice (Brinks *et al.*, 2007) to name but a few. Several behavioural animal models of stress-sensitivity such as the swim low-active model (SwLo), the learned helplessness model and the chronic mild stress model (CMS) have also been documented (Yadid *et al.*, 2000). Due to the close association between mood and anxiety disorders, these rat and mouse lines have been shown to be stress-sensitive, relative to controls. Besides that these lines display behavioural similarities with well-described characteristics of the human condition (known as face validity), some have also been shown to exhibit neurobiological dysfunction corresponding to that observed in humans suffering from MDD (construct validity), as well as to respond similarly to drugs used to treat depression in humans (predictive validity).

The current study in stress-sensitive rats focuses on the effects of early-life exposure to MA on behaviour (in particular as related to depression) later in life. Therefore, this Chapter will describe major depression as a mood disorder (definition, aetiology & treatment), critical aspects of animal models of depression, general characteristics of neurodevelopment in the human versus rat brain, prenatal MA exposure and teratogenicity, as well as general properties and attributes of MA and its abuse.

2.1 Background and epidemiology of methamphetamine

2.1.1 History

Substance abuse is an age-old problem associated with decreased productivity, decreased quality of life and increased risk for developing additional health problems (Weich & Pienaar, 2009). The list of addictive substances continues to grow each year, with MA as a more recent addition. The impact on communities affected by abuse of MA can be devastating, resulting in increased pressure on the health care system, violent or drug-related crimes and in some cases, long-lasting incapacitation (Vos *et al.,* 2010).

MA is a central nervous system (CNS) stimulant with powerful psychoactive and addictive properties (Panenka *et al.,* 2013; Meredith *et al.,* 2005). The first documented synthesis of MA from ephedrine dates back to 1893 by the Japanese scientist Nagai Nagayoshi. In 1919 Akira Ogata was first to synthesise it in crystalline form. Years after being synthesised, it was first used in the 2nd world war by the two opposing sides, the Axis and Allied forces. The stimulant properties of MA rendered soldiers courageous and enhanced endurance during stressful long hours of battle associated with sleep- and food deprivation (Meredith *et al.,* 2005; Stedham, 2007). MA was also employed to motivate soldiers to embark on perilous missions often assigned to them. Medical use for MA originally related to obesity, asthma, attention-deficit hyperactivity disorder (ADHD), narcolepsy and, before the addictive properties were well publicised, as energisers for students studying through the night or for truck drivers driving for long uninterrupted periods, often after dark (Anglin *et al.*, 2000; Krasnova & Cadet, 2009; Stedham, 2007). Presently, MA is registered at the Food and Drug Administration (FDA) in the United States of America (USA) for the treatment of juvenile ADHD and treatment-resistant obesity. Although controversial, it can be legally prescribed in the USA and is marketed under the trademark name Desoxyn® (Chiaia-Hernandez *et al.*, 2011; DEA, 2011; Salocks & Kaley, 2003). In March 2006, however, the Combat Methamphetamine Epidemic Act was passed in the USA, making access to the precursor chemicals, especially ephedrine and pseudoephedrine, more controlled and secure (USA, 2005). This may explain the subsequent drop in MA-laboratory confiscations. This law on products containing pseudoephedrine limited illegal MA manufacturing in the USA, but it has not been very effective, as there were resulting increases in the smuggling of MA over the Mexican border. The Mexican drug cartels use the routes already established for cocaine to smuggle MA into the United States (UNODC, 2009). Currently, with its easy accessibility, affordability and potency, it poses a very serious public health threat in most countries around the globe.

According to the 2009 world drug report of the United Nations Office on Drugs and Crime (UNODC), confiscation of global amphetamine-type substances (ATS) amounted to between 230 and 640 metric tons in 2007 alone. Based on estimates of manufacture and reported confiscations, the global misuse of ATS is estimated to involve between 7% and 19% of the population. The amount of clandestine MA laboratories detained and reported to UNODC from 1998 to 2007 peaked in 2004, with a total of 17 853 laboratories, surpassing all other ATS laboratory confiscations (UNODC, 2009). ATS consumption estimates in Africa are vague due to the lack of recent and reliable data. In fact, the only valid estimates derived from the region of Africa come from Southern Africa (UNODC, 2011).

2.1.2 Epidemiology

In many other countries, including South Africa (SA), MA is an illegal drug. It is known colloquially as "ice", "speed", "chalk", "crank" and "tik", with the latter being most commonly used in SA (DEA, 2011). According to the UNODC world drug report of 2009 and 2011, abuse of stimulants in SA is dominated by MA and methcathinone ("CAT"). Methcathinone is derived from cathinone, an active ingredient of the flowering, evergreen shrub *Catha edulis* (colloquially Arabian tea, khat, qat or gat). The shrub is native to East Africa and the Arabian Peninsula where it has an established cultural use in many social situations (DEA, 2011). Primarily the leaves are chewed, but can also be dried and smoked. It produces similar, but less extreme central effects than MA (DEA, 2011). However, MA remains the primary substance of abuse for which South Africans seek treatment (UNODC, 2011). MA abuse in SA currently persists primarily in the Western Cape Province with a greater demand for treatment over the past decade (Leggett, 2003; Vos *et al.*, 2010; Weich & Pienaar, 2009). Statistics regarding MA abuse in South Africa are limited and reports of clandestine labs in Gauteng and the surrounding areas are greatly outnumbered by the amount found in the Western Cape (Leggett, 2003). The Western Cape Province has the most drug-related crimes in SA as recorded by the South African police department statistics (SAPS, 2011) and specifically Mitchells Plain is known for its gang-related activity and drug trafficking (Haefele, 2011). The rapidly spreading abuse of MA yields serious consequences for at-risk individuals and their families.

The street value of MA is considered much more affordable in comparison to other illicit stimulants such as cocaine and heroin. MA became known as a powerful drug with easy access and low cost, globally increasing the demand for MA on the street. It has consequently become one of the most widely abused drugs world-wide, second only to cannabis (Büttner, 2011; UNODC, 2011). MA is synthesised from commonly available precursor substances

pseudoephedrine or ephedrine. These are found in many common cold medicines, freely available as over the counter medicines in most countries. Furthermore, the synthesis of MA is fairly simple, so that it can be performed at home or in backyard factories (Leggett, 2003; Sulzer *et al.*, 2005). As a result, it has become almost impossible to control, further compounding its global abuse problem.

Most unlicensed MA laboratories can be found in a variety of domestic areas including bathrooms, trailers, empty garages, sheds or even minivans. Simple recipes for the synthesis of MA (a process colloquially referred to as "cooking MA") can be found on the Internet. Nevertheless, the typical process of preparing MA can be very dangerous for an amateur with no knowledge of the basic chemistry or without basic safety measures in place. For example, anhydrous ammonia, a fertiliser commonly used on farms, is one of the typical ingredients used to synthesise MA and can cause extreme burns when it comes in contact with the skin and eyes. Furthermore, it is toxic when inhaled or ingested and is a caustic and volatile chemical that forms combustible gases upon heating in air (ORICA, 2008). Therefore it should be kept away from any source of heat or ignition, a component present in every MA laboratory. Not only is clandestine MA production illegal, but there is concern for occupants in the nearby vicinity who are also at risk of exposure to the toxic chemicals used. This adds to the risks associated with the so-called MA epidemic (Anglin *et al.*, 2000; Morris, 2007; Salocks & Kaley, 2003).

Additional dangers that accompany MA misuse are CNS symptoms such as paranoia, hallucinations and delusions following chronic use and resemble psychoses seen in schizophrenic patients. Multiple studies suggest that MA dependence is linked strongly with the higher prevalence of psychoses observed in chronic users (Darke *et al.*, 2008; McKetin *et al.*, 2006; Vos *et al.*, 2010; Zweben *et al.*, 2004). The symptoms of withdrawal closely resemble depression and can linger for up to 12 months. In 2004, Zweben and colleagues conducted a large study on the psychiatric symptoms of individuals abusing MA. They found that depressed mood accounts for the most common symptom experienced by chronic users, the cause of which was not investigated by the study (Zweben *et al.*, 2004). The extent to which pre-existing major depression may have contributed to susceptibility to drug abuse in the first place, or whether depression may have been a mere consequence of the drug abuse is still a matter of debate.

Nevertheless, MA dependence has proven difficult to treat. Severe psychological impairment seems present in most cases of MA dependence, rendering treatment and remission for MA addicts virtually ineffective. The clinical picture is further clouded and complicated by the frequent co-morbidity of disorders such as depression and schizophrenia (Brecht *et al.*, 2000;

Maglione *et al.*, 2000; Meredith *et al.*, 2005; Vos *et al.*, 2010). Of even greater concern is that, although it is known that MA puts a developing foetus at risk of seriously compromised neural development as well as painful postpartum withdrawal, pregnancy has not been shown to deter its misuse in pregnant women.

2.1.3 Drug abuse and dependence

2.1.3.1 The mechanism and pathology of drug addiction

Addiction: "The state or condition of being dedicated or devoted *to* a thing, esp. an activity or occupation; adherence or attachment, esp. of an immoderate or compulsive kind." (Oxford English Dictionary, 2012a)

Dependence: "The relation of having existence hanging upon, or conditioned by, the existence of something else; the fact of depending *upon* something else." (Oxford English Dictionary, 2012b)

Addiction and dependence are terms describing two different relationship states with a substance. Addiction refers to a psychological state in which the individual has an abnormally strong urge to use the substance and exhibits compulsive drug-seeking behaviour. Dependence (also known as physical dependence) refers to a physiological state in which the individual presents with withdrawal symptoms once the substance is withdrawn. This can be viewed as merely a result of adaptation in response to repeated exposure to the substance (O'Brien *et al.*, 2006). Arguably, an individual can be addicted but not dependent on a substance and vice versa.

Addictive substances such as MA, cocaine, 3,4-methylenedioxymethamphetamine (MDMA) and heroin all influence the neurocircuitry of the brain by releasing large (superphysiological) amounts of endogenous monoamines (Büttner, 2011; Meredith *et al.*, 2005). Under physiological conditions, behaviour deemed favourable for survival is reinforced by neurophysiological processes that constitute reward (e.g. pleasure), and those deemed unfavourable by processes that constitute penalty (e.g. pain or fear). Therefore, stimuli (experienced through sensory modalities) elicit appropriate biological reactions that can be experienced either as positive or negative. The reward centre in the brain processes these stimuli and provides feedback when they are encountered. This is termed positive or negative reinforcement and serves to encourage or discourage (and eventually alters) behaviour by responding with an appropriate affective state to the stimulus (DiChiara, 1995).

Most centrally active stimulants share the pharmacological property of promoting dopaminergic transmission, and depending on its pharmacological class, either via direct or indirect release of

DA from storage vesicles. In animals the basic needs for survival, such as food, water and reproduction (physiological needs, safety, love, esteem and need for self-actualization in humans, according to Maslow's theory of human motivation), are termed conventional motivators and these needs have been demonstrated to facilitate the release of DA in patterns correlating with what is understood as motivated behaviour. The mesolimbic pathway is implicated as one of the neuronal pathways utilised in motivated behaviour (Thrash *et al.*, 2010). Most addictive substances use the same neuronal pathways as do conventional motivators, for the purpose of rewarding the body with a positive affective state (Barr *et al.*, 2006; Di Chiara, 1995; Maslow, 1958). The mesolimbic reward pathway can be stimulated by MA, resulting in positive (rewarding) feelings of euphoria and excitement even in the absence of conventional motivators. This contributes greatly to the development of MA addiction (Thrash *et al.*, 2009).

2.1.3.2 Signs and symptoms of methamphetamine abuse

MA addicts normally exhibit signs of both addiction and dependence. The effects of a sudden increased activity in DA synapses account for feelings of confidence, alertness, control, hypersexuality and invincibility. The most common CNS effects of MA in moderate doses include euphoria, increased sexual drive, behavioural disinhibition, decreased appetite and a short-lived improvement in cognition (reaction, memory and clarity of thought) (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005). Negative effects such as anxiety, agitation, panic and paranoia are also common in moderate doses. High doses elicit psychoses such as excited delirium, hallucinations and delusions in some MA addicts and these symptoms can recur in chronic users even without the use of MA (Cruickshank & Dyer, 2009). As the effects of the drug wear off, addicts administer another dose to compensate for the lack of effect. This can go on for days and is termed binge-dosing, usually followed by a period of abstinence (Nordahl *et al.*, 2003).

The aftermath is termed the "crash" and typically manifests as lethargy, marked hypersomnia, irritability, severe dysphoria, anxiety and intense cravings for the drug (Meredith *et al.*, 2005). MA withdrawal induces a state of anhedonic dysphoria as the primary cause of cravings for the drug (Anglin *et al.*, 2000; Meredith *et al.*, 2005). Additional cravings can also be invoked by conditioned cues, namely independent occurrences or stimuli that were present during a euphoric episode of MA abuse. The strength of the induced cravings often dictates a relapse to MA abuse following MA abstinence (Anglin *et al.*, 2000). Furthermore, the withdrawal symptoms specific to chronic MA abuse are more severe and longer lasting than those seen in, for example, cocaine withdrawal. In fact, MA withdrawal symptoms may last up to 12 months (Cruickshank & Dyer,

2009; Meredith *et al.*, 2005). When undergoing rehabilitation addicts are usually overcome by intense cravings long before any successful rehabilitation can ease the distress of withdrawal (Anglin *et al.*, 2000). The degree and duration of MA-induced euphoria makes it a perfect escape from feelings of depression, guilt, lethargy and antisocial behaviour. In addition, the anticipation of withdrawal symptoms spurs a vicious cycle of abuse and may in part explain binge-dosing patterns observed in MA addicts (Meredith *et al.*, 2005). Consequently, physical dependence and psychological addiction, together with the ease of access to the drug, renders MA abuse an extremely difficult sociological challenge to overcome.

2.1.4 Adverse effects and toxicity of methamphetamine abuse

Concomitant substance abuse with MA poses an even greater health risk. Most substance abusers tend not to limit themselves to one drug (Barr *et al.*, 2006; Brecht *et al.*, 2000; Büttner, 2011). Several other addictive substances such as cocaine, opiates, alcohol and cannabis are abused concomitantly with MA, which severely increases the toxicity of the concurrently abused substances (Darke *et al.*, 2008; Kenneth & Geerts, 2007).

Vasoconstriction of coronary arteries and coronary thrombosis are effects of MA use. Chronic use is associated with ventricular hypertrophy, a condition that can predispose an individual to a serious myocardial event (Darke *et al.*, 2008; Meredith *et al.*, 2005). Since MA is known to increase heart rate and to cause vasoconstriction, thereby increasing blood pressure and myocardial oxygen demand, concomitant use of alcohol can potentiate these cardiotoxic effects, rendering the combination deadly. Likewise, the concomitant use of MA and cocaine can increase the vasoconstrictive and cardiotoxic effects of both of these drugs (Darke *et al.*, 2008; Meredith *et al.*, 2005). Whereas the opioids cause respiratory depression, MA increases the oxygen demand of the heart, rendering the combination highly cardiotoxic (Darke *et al.*, 2008). MA elicits peripheral effects such as an increase in body temperature, palpitations, fast breathing, elevated blood pressure, dilated pupils and excessive sweating (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005). The peripheral effects are usually relied upon to recognise a MA addict under the influence.

The preferred route of administration of MA abuse (particularly amongst frequent users) is the intravenous route. This delivers the drug directly into the systemic circulation, producing a rapid onset of action and an intense euphoric effect (Darke *et al.*, 2008). It produces a faster and more intense rush and following such an intense peak in plasma levels, it is not surprising that the negative symptoms are also experienced more intensely (Domier *et al.*, 2000). Intravenous injection as route of administration increases the risk of contracting a cohort of blood-borne

diseases, such as HIV-AIDS (human immunodeficiency virus-acquired immunodeficiency syndrome) and hepatitis C. This most commonly occurs with the sharing of needles or engaging in risky sexual behaviour commonly seen with MA addicts (Büttner, 2011; Cruickshank & Dyer, 2009; Darke *et al.*, 2008; UNODC; 2011). Alternatively, smoking of the drug is also associated with high bioavailability and a fast onset of action, yet being less invasive, thereby rendering popularity also to this route of administration (Meredith *et al.*, 2005). Oral, intra-nasal, pulmonary and intravenous routes of administration ultimately all lead to the same pharmacological effects once absorbed. However, the time until onset of action and peak plasma levels differ, creating subtle differences in experience (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005 Nordahl *et al.*, 2003). Tolerance to the drug develops with chronic use, requiring increasingly larger doses to experience the same euphoria as with the initial administrations (Anglin *et al.*, 2000). The metabolism of the drug does not change with chronic exposure and it is suggested that alterations in neurochemistry (and hence pharmacodynamic tolerance) rather than pharmacokinetic tolerance lead to dose escalation (Cruickshank & Dyer, 2009).

Physical symptoms of MA overdose include nausea, vomiting, chest pain, increased heart rate and body temperature, tremors, dilated pupils and irregular breathing. Seizures are uncommon but have been documented with overdosing (Cruickshank & Dyer, 2009 Darke *et al.*, 2008). Determining a lethal plasma concentration of MA has proven difficult, because of individual variations in metabolism, as well as the development of tolerance. Plasma concentrations as low as 90 μg/ml have proven fatal, although survival at concentrations as high as 9 460 μg/ml have been documented (Cruickshank & Dyer, 2009). MA overdose is not commonly listed as the cause of death, but rather the circumstances created by abuse of the drug such as suicide, organ failure due to chronic use, car accidents while driving under the influence and diseases or infections acquired from unsterilized injection needles used to administer MA (Darke *et al.*, 2008; Meredith *et al.*, 2005; UNODC, 2011).

2.2 Major depressive disorder

2.2.1 Definition and diagnosis

MDD is one of the most prevalent psychiatric disorders worldwide, and is regarded as one of the leading causes of disability and disease (Richards, 2011). Its high prevalence and significant impact on economy ensures that it enjoys a very high priority in policies of mental health management.

The Diagnostic and Statistical Manual of mental disorders, 4th ed. (DSM-IV), currently defines the criteria of MDD as follows (APA, 1994):

A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure

(1) depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g. feelings of sadness or emptiness) or observation made by others (e.g. appears tearful). Note: in children or adolescents it can be irritable mood
(2) markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others)
(3) significant weight loss when not dieting or weight gain (e.g. a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day. Note: in children, consider failure to make expected weight gains
(4) insomnia or hypersomnia nearly every day
(5) psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down)
(6) fatigue or loss of energy nearly every day
(7) feelings of worthlessness or excessive inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick)
(8) diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others)
(9) recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan or a suicide attempt or a specific plan for committing suicide.

B. The symptoms do not meet the criteria for a Mixed Episode.

C. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

D. The symptoms are not due to the direct physiological effects of a substance (e.g. a drug of abuse, a medication or a general medical condition such as hypothyroidism).

E. The symptoms are not better accounted for by bereavement i.e. after the loss of a loved-one, the symptoms persist for longer than 2 months or are characterized by marked functional impairment, morbid preoccupation with worthlessness, suicidal ideation, psychotic symptoms,

or psychomotor retardation.

Common characteristics of MDD are summarised as a loss of interest or pleasure in previously enjoyable activities, feelings of guilt or low self-worth, changes in appetite and/or sleep patterns, impairment in concentration and memory and low energy levels (O'Donnell & Shelton, 2012; Richards, 2011).

The prevalence of MDD is increasing at an alarming rate. Projections of the World Health Organisation (WHO), suggest that MDD will become the 2nd highest cause of global burden by 2020 (Andersen, 2003; WHO, 2012). Efforts to develop safe and effective anti-depressants became an area of focus for much research. However, of all the contributing factors, drug abuse tends to be highly prevalent amongst individuals suffering from depression and may exacerbate the already serious disorder (Anglin *et al.*, 2000; Meredith *et al.*, 2005). Besides the well-documented positive correlation between drug abuse and mood disorders, drug withdrawal-induced depression is also not a novel concept (Renoir *et al.*, 2012).

2.2.2 Aetiology of depressive disorder

Both hereditary factors (genetic predisposition) and environmental factors (e.g. chronic stress or drug abuse) has been shown to contribute to the eventual manifestation of MDD. Therefore, a combination of enhanced vulnerability and major adverse life events is required for the development of the disorder (Harro, 2010). In addition, MDD is commonly a comorbidity of a spectrum of psychiatric disorders. In this regard, both the genetic predisposition and the environmental factors associated with the development of MDD are also associated with the development of other psychiatric disorders, such as anxiety-related disorders. In fact, when comparing the similarities between symptoms and risk factors of MDD and anxiety-related disorders, one can conclude that the underlying neurobiological mechanisms may also be related (Harro, 2010).

Genetic predisposition to certain affective disorders has been suggested and supported by multiple studies (Andersen, 2003; Harro, 2010; Swaab *et al.*, 2005). Considering the difficulty of isolating such genes and proving their role in any disorder, one has to make assumptions based on case studies and animal models. Studies on monozygotic twins provide us with an outlook based on genetics and although the similarities are remarkable, they fall significantly short of 100% (Andersen, 2003) The heritability index for depression is 70% as suggested by twin studies, although no single gene has been identified (Andersen, 2003; Schatzberg, 2005). In

fact, there is no indication that there is only one neurobiological type of depression, so that various genetic haplotypes and/or neurobiological phenotypes may be involved.

A number of studies have suggested that a stressful environment plays an important role in the development of depression along with several identified risk factors. The early stages of neurodevelopment may be especially vulnerable to influence because of the brain's ability to adapt to its environment when exposed to different elements (Andersen, 2003; Murrin *et al.*, 2007). Several lines of data support the fact that environmental influences play just as significant a role as congenital factors in the development of MDD (Andersen, 2003; Frost & Cadet, 2000; Renoir, 2012). In this regard, brain maturation can be altered by both quantitative and qualitative environmental stimuli, depending on the timing and magnitude of the insult and the brain region of interest (Andersen, 2003).

2.2.2.1 Hypotheses of the neurobiological basis of depressive disorder

The discovery of the first anti-depressants in the 1950s revolutionised the understanding of psychiatric disorders and gave birth to hypotheses (and associated research) into the idea that MDD may indeed have a neurobiological basis (López-Muñoz & Alamo, 2009). In fact, many elucidations concerning the neuropathology of MDD is based on the neurobiological pathways affected by effective antidepressants. All currently used antidepressants modulate monoaminergic neurotransmission. The earliest ones, namely the monoamine oxidase inhibitors and the tricyclic antidepressants, and even the selective serotonin reuptake inhibitors, all elicit their effects by an initial elevation of synaptic monoamine (DA, NE or 5HT) levels. The original catecholaminergic hypothesis of depression followed the serendipitous discovery of antidepressants, predicting that depleted levels of catecholamines (particularly NE) contribute to the pathology of the disorder (Schildkraut, 1965). The discovery of monoamine reuptake transporters provided a new target for the development of antidepressants. Increased monoamine levels can be elicited by inhibiting these reuptake mechanisms (Iversen, 2006).

Different hypotheses of the neurobiological basis of depression have been suggested over the years, building on the catecholamine hypothesis. These include the monoamine hypothesis (López-Muñoz & Alamo, 2009; Sapolsky, 2000), the cholinergic super sensitivity hypothesis (Dilsaver, 1986; Janowsky *et al.*, 1972), the hypothalamic- pituitary-adrenal (HPA) axis hyperactivity hypothesis (Ehlert *et al.*, 2001; Holsboer *et al.*, 2001), the neuroplasticity hypothesis (Manji *et al.*, 2001; Pittenger & Duman, 2008), the metabolic encephalopathy hypothesis (Harvey, 2008), the epigenetic modifications hypothesis (Wegener *et al.*, 2010) and the circadian rhythm hypothesis of depression (Germain & Kupfer, 2008).

2.2.2.2 Prevalence and socio-economic impact of major depressive disorder

Mental disorders such as schizophrenia, bipolar disorder and MDD are listed among the 20 leading global causes of disability (WHO, 2012). Severe depression is the leading cause of disability in both men and women, and is classified as a severe disability in category VII (along with active psychosis, severe migraine, quadriplegia and terminal stage cancer). Women of all income-types are twice as likely to develop MDD in comparison with men (WHO, 2012).

Lifetime prevalence rates for MDD in women and men have been reported as 10-25% and 5-12%, respectively (Oquendo *et al.*, 2004; Schatzberg, 2005; Yadid *et al.*, 2000). Inpatient and outpatient care comprise up to 18% of direct costs of treating MDD and the total sales of the selective serotonin reuptake inhibitors (SSRIs) in 2003 was in excess of $17 billion worldwide (Iversen, 2006; Yadid *et al.*, 2000). MDD is a considerable burden on the resources of the health care sector and the economy. In the USA in 2001, the economic burden of depression totalled $83.1 billion and 62% of this amount is related to workplace costs. In Europe in 2004, the annual cost of depressive disorders amounted to €118 billion. Currently, MDD affects the lives of approximately 350 million people globally (WHO, 2012). In fact, the overall health care cost of depressed patients is higher when compared to patients without depressive disorder and patients with other illnesses (Richards, 2011). Due to the high comorbidity rate with anxiety-related disorders and treatment-resistant forms of MDD, the need for effective and safe treatment continues to take priority in psychiatric drug development.

Untreated episodes of MDD typically last 6 months or longer, remission of symptoms follows in the majority of cases and function returns to a premorbid level. A significant proportion of the cases (20-30%), however, may retain persistent symptoms that do not meet the criteria for MDD. These symptoms can last for months or years and may be associated with some level of disability (APA, 1994). It is thus considered an enormous setback for overall productivity and quality of life (WHO, 2012; Yadid *et al.*, 2000). Risk factors to develop MDD have been identified but have not yet enabled effective global strategies to prevent this disorder.

Diagnosis, treatment initiation and treatment success is also suboptimal, with a significant proportion of under-diagnosis and delay of treatment initiation, delayed onset of therapeutic effect following initiation of therapy, intolerable side-effect profiles of treatments and treatment resistance (Nemeroff, 2007; Pampallona *et al.*, 2002).

2.2.3 General treatment of depression

2.2.3.1 Classification of antidepressants

Pharmacological intervention of MDD has been evolving since the discovery of the first antidepressant and many hypotheses have been formed on the basis of the mechanism of action of these agents (Harro, 2010; O'Donnell & Shelton, 2012). Many successful antidepressants have been developed to target monoaminergic neurotransmission, for example via inhibition of the reuptake mechanisms of monoamines, such as the dopamine transporter (DAT), the norepinephrine transporter (NET) and the serotonin transporter (SERT) (Iversen, 2006).

The first anti-depressant class developed from iproniazid, a drug designed for the purpose of treating tuberculosis (O'Donnell & Shelton, 2012). A notable side-effect of the drug was the elevated mood observed in the treated patients. Further investigation revealed it to be an inhibitor of monoamine oxidase (MAO) type A and B (O'Donnell & Shelton, 2012). MAO type-A preferentially deaminates epinephrine, NE and 5HT, whereas MAO type B preferentially metabolises benzylamine and phenylethylamine. Tyramine and DA are deaminated by both MAO-A and -B (medicinescomplete.com, 2013). A brief overview of the classification of antidepressants is discussed below and summarised in Table 2-1.

Table 2-1: Summary of current classification of clinical antidepressants

TCA	amineptine amitriptiline amoxapine clomipramine desipramine dibenzepin dosulepin doxepin	imipramine lofepramine melitracen noritriptiline opipramol pipofezine protriptiline trimipramine	**Bicyclic** viloxazine	**Tetracyclic** maprotiline mianserin mirtazepine
MAOI	iproniazid isocarboxacid	phenelzine tranylcypromine	**MAOI-A** moclobemide	**MAOI-B** selegiline
SSRI	citalopram escitalopram fluoxetine fluvoxamine	paroxetine sertraline vilazodone		

SNRI	desvenlafaxine duloxetine	milnacipran venlafaxine		
NARI	atomoxetine reboxetine			
NDRI	buproprion			
Atypical	agomelatine benzactizine lithium oxitriptan nefazodone	tianeptine trazodone tryptophan pirlindole setiptiline		

Tricyclic antidepressants (TCAs), the first prominent class of antidepressants, were derived from analogous structures of phenothiazine. These substances were developed for potential use as antihistamines, antipsychotics, analgesics and sedatives such as promethazine. Further development resulted in the synthesis of imipramine, which exhibits improved antidepressant activity, but without anti-psychotic activity (López-Muñoz & Alamo, 2009). The tricyclic class of antidepressants is named after their three-ringed structure. They differentially inhibit the reuptake of NE and 5HT, plus display activity at adrenergic, muscarinic and other receptor types (O'Donnel & Shelton, 2012; Trevor *et al.*, 2005).

The monoamine oxidase inhibitors (MAOI) alleviate depressive mood by inhibiting the metabolism of NE, DA and 5HT. In comparison with the safety profiles of other antidepressant classes they have a higher risk of developing life-threatening side-effects. MAOIs pose such a risk if used with tyramine-containing foods, such as cheese, milk and liver. Tyramine releases NE and if not metabolised, it can cause dangerous rises in heart rate and blood pressure, leading to cardiovascular complications and a hypertensive crisis (medicinescomplete.com, 2013; Sulzer *et al.*, 2005). The MAOIs are still used clinically in the treatment for MDD, although safer drug treatments have been developed.

SSRIs are the first class of antidepressants developed by rational drug design (López-Muñoz & Alamo, 2009). The resulting molecules selectively target the SERT to elevate 5HT levels while they also lack activity at cholinergic receptor types. Consequently they are free of many of the undesirable effects of the TCAs. The selectivity of the SSRIs for 5HT re-uptake renders them with a wider safety margin, making them suitable for patients at risk of overdosing, such as children, juveniles and geriatric patients. Fluoxetine was the first SSRI to be marketed

worldwide for depression, and is presently still one of the most widely prescribed antidepressant drugs (Iversen, 2006, López-Muñoz & Alamo, 2009).

Antidepressant drugs that selectively target the reuptake of both 5HT and NE have also been identified and named the serotonin-norepinephrine reuptake inhibitors (SNRIs) (e.g. duloxetine, venlafaxine) along with selective inhibitors of NE uptake (NARIs) (e.g. reboxetine, atomoxetine) (López-Muñoz & Alamo, 2009). 5HT receptor antagonists such as trazodone and nefazodone target the $5HT_2$ receptor family along with other receptors and are also effective as antidepressants. Agomelatine is a $5HT_{2C}$ receptor antagonist as well as an agonist at M_1 and M_2 receptors and acts in the superchiasmatic nucleus and pineal gland to re-entrain circadian rhythms (Millan *et al.*, 2003). Buproprion, a drug previously classified as atypical, is an inhibitor of NE and DA reuptake mechanisms, now referred to as a NE and DA reuptake inhibitor (NDRI). Atypical antipsychotics, especially olanzapine and quetiapine, are also used in combination with SSRIs and SNRIs for treatment-resistant depression without psychosis (Hamon & Blier, 2013). Interestingly, amphetamine-like substances were discovered to also weakly inhibit MAO (for extended review, see Sulzer, *et al.*, 2005).

2.2.3.2 Current treatment guidelines

Correct diagnosis of MDD is critical for the initiation of effective treatment. Dysthymic disorder, bipolar disorder, substance induced depressive disorder and other co-morbid disorders with overlapping symptomatology, such as cancer, can all influence the final diagnosis and treatment. Therefore, whereas MDD may be the primary diagnosis, it may also be a secondary diagnosis to another disorder that affects mood. Misdiagnosis may also result from inappropriate differential diagnosis, for example in patients with hyper- or hypothyroidism. Although most correctly diagnosed patients respond to antidepressant treatment, at least partially, roughly 20% of patients remain resistant to therapy and retain depressive symptoms even when treated with multiple antidepressants at adequate doses (O'Donnel & Shelton, 2012).

Non-pharmacological treatment strategies of MDD are used to supplement the use of antidepressants. These treatment strategies could include one or more of the following: changes in lifestyle such as exercise, nutrition and diet, meditation, relaxation exercises (recreational activities, stress management & activities, etc.), psychotherapy (regulating emotions, problem solving, social competence), disease management programs, electroconvulsive therapy, transcranial magnetic stimulation, deep brain stimulation, vagus nerve stimulation and sleep deprivation therapy, to name the most prominent (Fehlinger *et al.*, 2013; Nemeroff, 2007; Oestergaard & Møldrup, 2011).

Pharmacological treatment (drug treatment) includes a variety of antidepressants used in monotherapy or combination therapy. The most commonly prescribed antidepressants are the SSRIs, because their safety profile and tolerability surpass that of the older drugs. The SNRIs and NARIs are also widely prescribed (O'Donnel & Shelton, 2012). Older strategies involve treatment with a TCA or MAOI, though numerous side-effects cause patients to find these treatments hard to tolerate. Fortunately, the treatment options for MDD is expanding and new antidepressants include triple (NE, 5HT and DA) reuptake inhibitors (e.g. amitifadine), dual serotonergic-melatonergic drugs (e.g. agomelatine), drugs that act as 5HT modulators and simultaneous 5HT reuptake inhibitors, antagonists of CNS peptides (e.g. substance P antagonists), modulators of cyclic nucleotide signaling (e.g. phosphodiesterase inhibitors), σ_1 and σ_2 receptor ligands (e.g. (+)pentazocine, 1,3-di-o-tolylguanidine (DTG)), melatonin receptors dualists (e.g. M_1 and M_2 non-selective agonist ramelteon) and glutamate receptor antagonists (e.g. ketamine) (Ekmekcioglu, 2006; Gobbi & Blier, 2005; Krystal *et al.*, 2005; Maurice & Su, 2009; O'Donnel & Shelton, 2012; Sateia *et al.*, 2008; Wong *et al.*, 2006).

The therapeutic effect for all currently available antidepressants is not immediate, which may warrant extra caution in the first 3-4 weeks after treatment initiation, especially when the risk of suicide is greatest (Blier & Montigny, 1983; Willner *et al.*, 2013). Furthermore, failure to adhere to prescribed medication because of initial lack of effectiveness or other reasons can undermine the therapeutic efficacy of long-term treatment of MDD (Fava & Offidani, 2011; Willner *et al.*, 2013). Whereas some patients experience complete remission with the use of a single antidepressant, others may experience a partial remission after which additional antidepressants or an atypical antipsychotic may be added to the primary treatment (Connolly & Thase, 2011). Upon complete remission of symptoms a 6-12 month maintenance treatment phase is typically prescribed. The maintenance treatment prevents relapse and afterwards the drug is gradually withdrawn to avoid withdrawal (O'Donnel & Shelton, 2012). MDD has a wide spectrum of severity and a high frequency of comorbidities. Additionally, treatment dosage and –duration as well as adherence are problematic even when correct diagnoses are made. A high percentage of patients discontinue their antidepressant use prematurely or inappropriately, leading to a withdrawal syndrome. Discontinuation of therapy before the course is completed often happens because patients feel better and think that they no longer need the treatment. Also, intolerable side-effects may discourage continuing antidepressant use and if patients are not warned about the delayed onset of action, many stop taking it, thinking it to be ineffective. Discontinuation syndrome is documented with all antidepressant types (Blum *et al.*, 2008). Sudden discontinuation of antidepressant treatment results in withdrawal symptoms distinct to each

antidepressant type. SSRIs are documented to produce dizziness, insomnia, nervousness, agitation and nausea whereas TCAs produce anorexia, chills, diaphoresis, fatigue, weakness, malaise and vomiting. To minimise the effects of sudden discontinuation, the treatment is tapered off (Bosker *et al.*, 2010; Rosenbaum *et al.*, 1998).

Although the use of combined antidepressant treatment may be beneficial (de Maat *et al.*, 2007; Pampallona *et al.*, 2002) it may also result in severe and sometimes life threatening side-effects such as serotonin syndrome (Sternbach, 1991). Serotonin syndrome is caused by an excess of serotonergic activity at central receptors. When two drugs that increase serotonergic activity in the CNS are combined, the result is agitation, ataxia, diaphoresis, diarrhoea, fever, hyperreflexia, myoclonus, shivering and changes in mental status (medicinescomplete.com, 2013). The primary treatment for serotonin syndrome is cessation of all serotonergic drugs, administration of non-selective serotonin antagonists and supportive measures (O'Donnel & Shelton, 2012). It is especially MAOIs that are contra-indicated for use together with TCAs, SSRIs and buproprion. When discontinuing the use of a MAOI and starting another serotonergically active drug, a minimum period of 14 days must lapse between taking either of the drugs. This provides enough time for the MAOI to wash out of the system.

2.2.4 Animal models of depression

Clinical testing of drug treatments in humans is very practically challenging and associated with many ethical obstacles. For instance, environmental and psychological factors that may interfere with test results in human trials, such as diet, stress, placebo responses, self-medication and daily activity cannot always be controlled. Invasive studies are almost impossible and therefore a very limited amount of biological information can be extracted from human studies. Animal subjects that serve as a substitute/model for the human condition is a much more practical and valuable approach, since it allows for experimentation under controlled conditions, while at the same time affords the opportunity to perform corresponding behavioural and neurochemical studies. A model is defined as an experimental preparation/animal developed for the purpose of studying a condition in the same or in a different species (Yadid *et al*., 2000). Therefore, the objective of an animal model as surrogate for a psychological disorder in humans is to replicate the closest possible environment of the human disorder under controlled experimental conditions. Selective rodent models with specific traits that mimic the human condition can be used in these instances if the standards of face, construct and predictive validity (see below) are met (Barr *et al.*, 2006; Neumann *et al.*, 2011). Previous studies have indicated in more than one area of study that animal models provide a solid basis for producing and reproducing results that can be used in

furthering the study of specific medications or treatments pertaining to human disorders (Overstreet *et al.*, 2005) and also to allow for deeper mechanistic studies to be undertaken in order to better understand the pathology of the illness.

Several animal models based on increased stress-sensitivity have been developed for studying the neurobiology and behavioural characteristics of MDD (Yadid *et al.*, 2000). These models include both environmental and genetically susceptible subjects such as: olfactory bulbectomised rodents (Cairncross *et al.*, 1977; Cairncross *et al.*, 1978), isolation-induced hyperactivity (Sahakian *et al.*, 1975; Sahakian & Robbins, 1977), separation models (Katz, 1981; McKinney & Bunney, 1969; Reite *et al.*, 1981), behavioural despair (Porsolt, 1978a; Porsolt, 1978b), chronic unpredictable stress (Katz *et al.*, 1981; Katz & Siebel, 1982), intra-cranial self-stimulation (Barrett & White, 1980; Kokkinidis & Zacharko, 1980; Simpson & Annau, 1977), learned helplessness (Maier, 1984; Maier & Seligman, 1976), chronic mild stress (CMS) (Willner, 1997), Swim Low-Active (SwLo) line rats (Weiss *et al.*, 1998) and FSL rats (Overstreet, 1993).

2.2.4.1 Face validity

Face validity of an animal model is defined as the extent to which the animal model resembles the symptomatic appearance of the psychiatric condition (Malkesman & Weller, 2009; Yadid *et al.*, 2000). When a behavioural trait of an animal mimics a corresponding behavioural dysfunction in humans, this is demonstrative of face validity for the disorder under study. Increased time spent immobile in the FST, interpreted as "behavioural despair", weight-loss, sleep-disturbance and decreased social play are a few of the characteristics observed in a rat model of depression. The characteristics observed in humans with MDD are similar to those observed in the stress-sensitive rat, except for suicidal ideation which cannot be measured in animals. The forced swim test paradigm remains the more robust measurer of depressive-like behaviour in rats (Malkesman & Weller, 2009).

2.2.4.2 Construct validity

Construct validity of an animal model is defined as consistency of the animal model with the human counterpart in terms of the neurobiological basis or theoretical rationale of the condition (Malkesman & Weller, 2009; Yadid *et al.*, 2000). For instance, stress-sensitive FSL rats are hypercholinergic, making them more responsive to a cholinergic challenge, and presenting with higher levels of blood cortisol and decreased monoamine levels. The cholinergic hyperactivity hypothesis of depression is well recognised (Janowsky *et al.*, 1972) while similar neurohormonal deficiencies as that noted in the FSL rats are also observed in depressed humans (Overstreet *et al.*, 2005). However, construct validity is less reliable when an animal model is used to study a

human condition. The physiology alone differs in many aspects that are important when translating the research for human use (e.g. cholinergic activity). However, mood disorders contain a psychological factor (i.e. thoughts of suicide, anhedonia) that is not measurable in the FSL rat (Overstreet *et al.*, 2005).

2.2.4.3 Predictive validity

Predictive validity is defined as the extent to which the animal model favourably responds to the same drugs as humans with the same condition (Malkesman & Weller, 2009; Yadid *et al.*, 2000). A notable decrease in depressive-like behaviour in response to antidepressant treatment is taken as confirmation of predictive validity of the animal model. Therefore, they respond in the same way to drug therapy as depressed individuals would. Drugs that have no effect in depression in both humans and FSL rats are also documented, such as amphetamine and scopolamine (Overstreet *et al.*, 2005). Furthermore, drugs that are effective in adolescent depression (e.g. venlafaxine) are not effective in childhood depression (Emslie *et al.*, 2007). Similar results were documented in FSL and Flinders Resistant Line (FRL) rats (Steyn, 2011).

2.2.5 Flinders sensitive line rat model of depression

As discussed in section 2.2.2 many psychiatric disorders are believed to result from a combination of heritable and environmental factors. Therefore, to induce psychiatric-like disorders in animals by only introducing an adverse environmental factor (stressor) may not optimally reflect the human condition. Rather, an animal model of a particular psychiatric disorder may be better representative of the human condition if a genetic predisposition also underlies its manifestation (Malkesman & Weller, 2009; Overstreet *et al.*, 2005).

Hence, selective breeding of rats to display recognised traits of depression has proven very useful, thereby introducing genetic susceptibility in the animal model. Genetic variability or inter-individual differences can arguably alter a person's susceptibility to develop a certain disorder, including developing the disorder following a particular environmental stressor as precipitating trigger (Neumann *et al.*, 2011; Pittenger & Duman, 2008). The FSL rat is one example of selective breeding to yield stress-sensitive animals and is a well-described and validated animal model of depression. In fact, ample data support face, predictive and construct validity of the FSL rat model (Malkesman & Weller, 2009; Harro, 2010; Yadid *et al.*, 2000). Adding to its usefulness, the normal FRL rat acts as a corresponding negative control, where genetic susceptibility is absent (Overstreet *et al.*, 2005). FSL rats were originally bred from Sprague-Dawley rats to be genetically resistant to diisopropyl fluorophosphate (DFP), an organophosphate anticholinesterase agent. The result was a breed of rats rendered more

sensitive to cholinergic challenges, unexpectedly also displaying depressive-like and anxiety-like behaviour which was explored further (Overstreet *et al.*, 2005). Indeed, cholinergic supersensitivity has been associated with MDD (Dilsaver *et al.*, 1986; Janowsky *et al.*, 1972), further supporting these findings in FSL rats. Subsequently the robust stress-sensitivity of FSL rats and their response to drugs effective as antidepressants in humans has been described by various laboratories using various behavioural screening tests (Auta *et al.*, 2000; Harro, 2010; Kotsovolou *et al.*, 2010; Malkesman & Weller, 2009; Overstreet *et al.*, 2005). Considering that predisposition to disorders can be controlled or factored into the equation in an animal model while it cannot be determined in human participants partaking in clinical trials, the value of an animal model in studying a disorder is immense.

Characteristics of the FSL rat that emphasises its vulnerability are neurochemical factors such as heightened constitutive nitric oxide synthase (NOS) activation following stress exposure, decreases in neuropeptide Y levels and, in humans and FRL rats but not in FSL rats, increased HPA axis reactivity cortisone levels following stress. Furthermore, raised catecholamine levels in certain brain regions are found in FSL but not FRL rats, with an uncertainty as to how this can be translated to human pathology (Neumann *et al.*, 2011; Wegener *et al.*, 2010).

Brain-derived neurotrophic factor (BDNF) has been documented to have a positive correlation between the severity of MDD and a reduction in BDNF levels (Aydemir *et al.*, 2006). Decreased BDNF levels in the hippocampus of the FSL but not the FRL rats are documented (Elfving *et al.*, 2010), supporting the rationale of measuring BDNF levels in an animal model of depression (Neumann *et al.*, 2011). Furthermore, cell proliferation is documented to be affected by antidepressant treatment and to differ in FSL and FRL rats (Chen *et al.*, 2010). Decreased neurogenesis in the hippocampus is linked to the aetiology of depression and is also documented in FSL rats, which provides support for the neuronal plasticity hypothesis of depression (see section 2.2.2.1) (Neumann *et al.*, 2011).

A list of putative endophenotypes can be used to describe MDD in an effort to overcome the methodological difficulties of elucidating a genetic basis for the disorder. Endophenotypes are hereditary characteristics associated with a condition but are not necessarily a symptom of the condition. Depressed mood is described as mood bias toward negative emotion and is symptomatic of MDD, and cannot be modelled effectively in rats. Anhedonia is described as a decreased ability to feel pleasure, a characteristic that presents as decreased sucrose preference and decreased intracranial brain stimulation in rats (Yadid *et al.*, 2000). Impairment in learning and memory is also associated with the manifestation of MDD although no definitive deficits are

observed in the FSL rat under basal conditions (Overstreet *et al.*, 2005). However, impaired executive cognitive function (response speed) and psychomotor retardation is modelled in the FSL rat by a reduction in bar-pressing for rewards and decreased activity in the OFT (Overstreet *et al.*, 2005; Yadid *et al.*, 2000). A change in appetite/weight is a common occurrence in depressed individuals and the FSL rats are documented to have a different feeding rate than control rats (Overstreet *et al.*, 2005; Yadid *et al.*, 2000). A high-fat diet is documented to exacerbate depressive-like behaviour in FSL rats, possibly in connection with co-morbid diabetes mellitus type 2 (Abildgaard *et al.*, 2011). A comparison of behaviour of depressed humans and FSL rats is presented in Table 2-2, emphasising the similarity in their behaviour. Of interest is that increased stress sensitivity is gender specific and is characteristic of women. Women are 50% more likely to develop depression than men (WHO, 2012). This correlates with gender-specific stress-sensitivity observed in female FSL rats (Bjørnebekk *et al.*, 2007).

Biological endophenotypes such as increases in rapid eye movement (REM) sleep, immune function abnormalities, abnormalities in brain structure and function (hippocampus), and abnormalities in neurotransmitters and corticotrophin-releasing hormone (CRH) are present in both depressed humans and FSL rats (Hasler *et al.*, 2004; Overstreet *et al.*, 2005; Yadid *et al.*, 2000).

Table 2-2: Comparison of behavioural characteristics of FSL rats and depressed individuals (Overstreet *et al.*, 2005).

Symptom/observation in depressed individuals	Behavioral measure in FSL rats
A. Core symptoms	
Suicidal ideation and/or attempt	Cannot be modelled
Psychomotor retardation	**Reduced bar pressing for rewards**
Anhedonia	*Normal ICSS threshold and Saccharin Intake*
Reduced appetite/loss of weight	**Lower body weight; reduced appetite**
Cognitive disturbance	*Normal accuracy in food-motivated task*
Elevated REM sleep	**Elevated REM sleep**
Reduced REM sleep latency	**Reduced REM sleep latency**
Reduced slow wave sleep	*Normal slow wave sleep*
B. Associated variables	
Reduced killer T cell activity	**Reduced killer T cell activity**
Other immune abnormalities	**Other immune abnormalities**
Higher Incidence of IBS	**Greater gut sensitivity to antigen**
Higher incidence of asthma	**Greater airway sensitivity to antigen**
Anxiety of some types	***Anxiety in some tasks***

Bold in both columns indicates a match; *italics in one column* and normal font in the other column indicates a mismatch.

2.3 Neurodevelopment and teratogenicity

2.3.1 Stages of neurodevelopment of the rat in comparison with man

Neurodevelopment, including that of the brain, is somewhat different between species. This can be seen in differences in development relative to the time of birth and lifespan. However, there are also remarkable similarities in terms of the overall pattern and sequence of development. In the rat the ratio of brain weight to total body weight at birth is similar to that of the human during the second trimester of pregnancy (Dobbing & Sands, 1979). Following hormonal changes at 5 weeks of age, sexual maturity is reached. This biological phase of puberty in the rat corresponds with that of adolescence in humans (Murrin *et al.*, 2007).

A similar pattern of neurodevelopment is described across all mammalian species. In this regard, the serotonergic system is one of the first to develop in the mammalian brain (Andersen, 2003; Gaspar *et al.*, 2003). Motor development precedes cognitive development and it parallels the ontogeny of the striatum and cortex, respectively. Neurotransmitters serve as a neurotrophic factor, thereby predisposing them to control their own development and growth (Andersen, 2003). Factors such as tissue or hormonal development that corresponds in rats and humans can be used to establish beacons/biomarkers in a biodevelopmental timeline and then to compare the human versus animal models. Hippocampal development in rats on postnatal days 11-20 correlates anatomically with hippocampal development in humans during the third trimester, where this brain region plays an important role in spatial learning and memory (Williams *et al.*, 2002).

Neurodevelopment in rats, as in humans, are affected by hereditary and environmental factors. The latter include both physical and psychosocial factors (Andersen, 2003; Buwalda *et al.*, 2013). Humans and rats are social beings and share a need for social interaction, and hence the social environment can greatly affect neurodevelopment (Buwalda *et al.*, 2013; de Jong *et al.*, 2005; Farrington, 1993). This susceptibility is clearly demonstrated by social isolation rearing of rats, where typically 21-day-old pups (just weaned) are separated from their mothers and siblings, and reared in isolation for 6-8 weeks. This leads to severe psychosocial stress, which in turn modulates neurodevelopment to result in altered levels and turnover ratios of biogenic amines (Trabace *et al.*, 2012); eventually this leads to behavioural, social and cognitive changes in adulthood (Möller *et al.*, 2013). Similar response to a lack of (or dysfunctional) social interaction early in life has been described in humans (Farrington, 1993). Based on these similarities in the response of rats and humans to the psychosocial environment, rats can be used to model responses to environmental interventions corresponding to that seen in humans.

2.3.2 Pre- and postnatal stages of monoaminergic development in the rat and human

Monoaminergic neurotransmitters include 5HT and the catecholamines (NE and DA). Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the synthesis of DA and NE by hydroxylation of the amino acid L-tyrosine to L-dihydroxyphenylalanine (L-DOPA). L-DOPA is then decarboxylated to DA (inside storage vesicles of the synapse) followed by hydroxylation by dopamine-ß-hydroxylase (DBH) to form NE (Figure 2-1, pathway "a") (Trevor *et al.*, 2005).

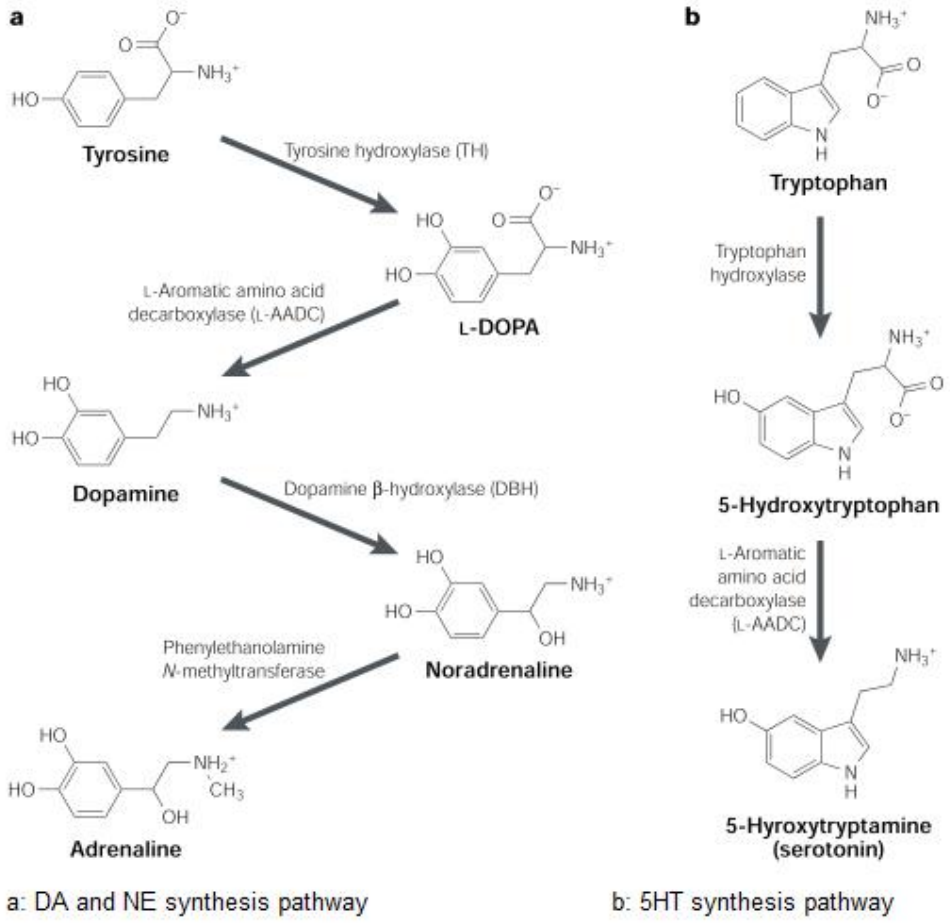

Figure 2-1: Synthesis pathways of the monoamines (Adapted from Goridis & Rohrer, 2002).

These neurotransmitters are stored in vesicles until released into the synapse by exocytosis. Tryptophan hydroxylase converts tryptophan, the precursor amino acid for 5HT, to 5-hydroxytryptophan, which is then further converted to 5HT (Figure 2-1). Termination of action of monoamines in the synaptic cleft occurs through reuptake mechanisms, diffusion out of the synaptic cleft and metabolism of the remaining molecules. The reuptake mechanism for 5HT, DA and NE respectively, is the SERT, DAT and NET. The vesicular monoamine transporter-2 (VMAT-2) is the mechanism that stores monoamines such as DA and NE in vesicles inside the neuron. The metabolising enzymes are MAO, subdivided into MAO-A and MAO-B) and

catechol-O-methyl transferase (COMT). MAO is also present on the mitochondria in the adrenergic nerve ending, metabolising a portion of the monoamines in the cytoplasm (Trevor *et al.*, 2005). Functions of 5HT, DA and NE, and their importance for homeostasis are discussed in the next section. The serotonergic, noradrenergic and dopaminergic neurodevelopment is discussed in this section and is summarised in Figure 2-2.

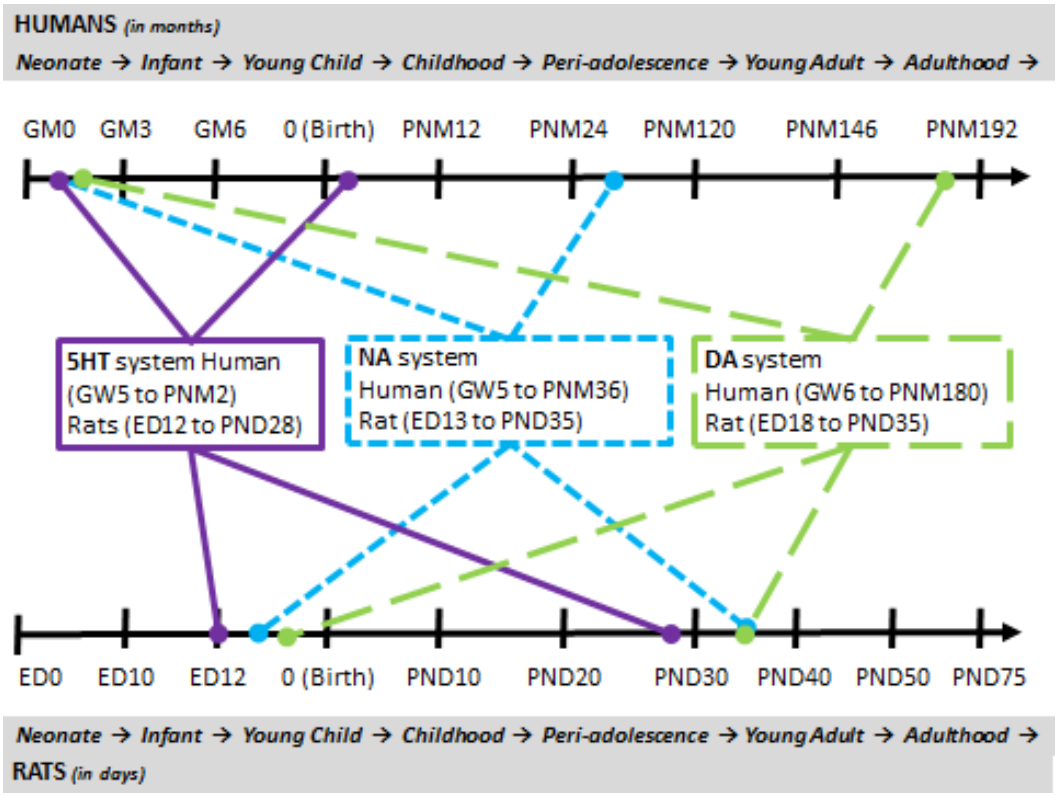

Figure 2-2: A schematic overview of the comparison of the neurodevelopment of serotonergic (5HT), noradrenergic (NE) and dopaminergic (DA) systems as they occur at various phases along a timeline in the human versus in the rat. Note the similarity in the over-all pattern of development over time. (GM-gestational months, PNM-postnatal months, GW-gestational weeks, ED-embryonic days, PND, postnatal days).

The serotonergic system in both rats and humans mature before the noradrenergic and dopaminergic systems (Murrin *et al.*, 2007). As summarised in Figure 2-2, human serotonergic cells and 5HT can be detected at 5-12 weeks of gestation and the hippocampus and cortex exhibit a prenatal peak in the density of $5HT_{1A}$ receptors at gestational weeks 16-22. The activation of these receptors is associated with increased neurogenesis, neural differentiation and dendritic maturation of the hippocampus (Andersen, 2003; Herlenius & Lagercrantz, 2004; Murrin *et al.*, 2007). Rat monoaminergic neurons can be detected by day 13-18 of gestation and 5HT levels in the rat brain peak at postnatal days 21-30, after which they decline somewhat to adult levels (Andersen, 2003; Murrin *et al.*, 2007). Serotonergic maturation in rodents is achieved postnatally and is documented to have an early and dynamic expression during

neurodevelopment (Gaspar *et al.*, 2003). Inadequate or over activation of 5HT receptors during early development may be involved in permanent alterations of neuronal pathways and therefore possibly a predisposition to develop anxiety disorders or drug addiction later in life (Gaspar *et al.*, 2003; Kirby *et al.*, 2011). Gaspar and colleagues extensively studied the development of the mouse serotonergic system and found that abnormalities in development of the somatosensory cortex in MAO-A knockout mice strongly correlate with serotonergic interference in the first postnatal week. No visible effects could be found with intervention during the embryonic or adult phase (Gaspar *et al.*, 2003).

TH can be detected by gestational week 4 in humans and NE by week 6. Human NE levels in the brain increase from 2 months of gestation onward. Noradrenergic neurons appear early in the rat at gestational days 10-14 and differentiation continues steadily in a nearly linear fashion until adulthood at approximately postnatal day 35-40 (Herlenius & Lagercrantz, 2004; Murrin *et al.*, 2007). Based on developmental studies of rhesus monkeys, 5HT and DA systems develop more rapidly than the NE system (Murrin *et al.*, 2007). Adrenergic transmission is possibly involved in regulating the generation, migration and maturation of cerebral cortical cells, most likely because blocking neurotransmission prevents astrogliosis and glial cell proliferation (Herlenius & Lagercrantz, 2004). DA is decarboxylated to form NE, therefore the development of the noradrenergic system can be expected to more closely follow that of the dopaminergic system than the serotonergic system. Catecholamines play a crucial role in early stages of development. This has been demonstrated by deleting genes encoding for TH and DBH (Herlenius & Lagercrantz, 2004).

Dopaminergic neurons appear earlier in females than males in both rats (gestational days 10-15) and humans (gestational weeks 6-8). DA turnover during the perinatal period is relatively high in comparison with adult levels (Herlenius & Lagercrantz, 2004). Synaptic density in the human frontal cortex decreases by 40% between ages 7 and 15 years, correlating with comparable dopaminergic receptor changes in the adult brain (Andersen, 2003). On gestation day 14 in the rat, dopamine transporter binding sites are detectable in the striatum (Won, *et al.*, 2001). DA, DA uptake sites and TH activity account for 10% of adult levels at birth and increase to approximate adult levels between 28-35 days of age and the DA receptor density (D_1 and D_2) reach adult density after an almost linear increase from birth (Andersen, 2003). The first signs of dopaminergic axons may appear early in gestation but are developed over a longer period of time. DA is described as a modulator of the structural and functional development of neural circuitry by acting through different receptors (Frost & Cadet, 2000).

Once the initial phase of innervation is complete for the respective neurotransmitter systems, apoptosis (programmed cell death) prunes approximately half of all neurons shortly before birth (Andersen, 2003). A second wave of overproduction and pruning appears later in life, during prepuberty. The toxicity of MA on mature monoaminergic neurons suggest that a similar (perhaps more pronounced) effect can be achieved on developing neural circuitry (Frost & Cadet, 2000).

2.3.3 Function and dysfunction: the monoaminergic systems

Both antidepressants and CNS stimulants ultimately elevate the concentration of monoamines in the brain, eliciting a mood elevating or euphoric response, respectively (Murrin *et al.*, 2007). It has been well documented that the abuse of CNS stimulants causes long-term dysfunction of the monoaminergic systems via neurodegenerative mechanisms. In addition, there is a positive correlation between the presence of a mood disorder and the development of drug dependence (Renoir *et al.*, 2012).

Dysfunctional levels of monoamines have long been implicated as neuronal factors in the pathology of depression whereas the non-neuronal cells in the brain are currently also being investigated as a possible cause (Fatemi *et al.*, 2004; Iversen, 2006; Rajkowska *et al.*, 1999). Glial cells are the non-neuronal cells of the CNS and play an integral part in the development of neurons and neuronal tissue. The glial cells consist of astroglial cells (also known as astrocytes) and microglial cells. Astroglia provide the neurons with nutrients and play a possible role in the process of information storage. Astroglia play an integral role in maintenance of the blood brain barrier, regulation of glutamate neurotransmission and metabolism of neurotransmitters (Büttner, 2011). Microglia are termed the macrophage cells and are the primary antigen presenting cells of the CNS, serving immune-like functions such as removing bacteria and foreign bodies (Kitamura *et al.*, 2010; Thomas *et al.*, 2008). These cells play an important role in the neurodegenerative mechanism of oxidative stress that is discussed in section 2.3.4.3.

2.3.3.1 The serotonergic system

The serotonergic system matures rapidly, reaching typical levels and morphology found in adults much sooner than the noradrenergic or dopaminergic systems (Murrin *et al.*, 2007). Serotonergic neurotransmission plays an important role in many brain functions, including sleep, cognition, sensory perception, motor activity, temperature regulation, nociception, mood, appetite, sexual behaviour, and hormone secretion (Leonard, 2003). Receptor subtypes for 5HT have been classified and identified to have functions in different parts of the brain and body (e.g. the CNS and the gastro-intestinal (GI) tract). 5HT is also taken up and stored in blood platelets

to assist with platelet aggregation (Sanders-Bush & Hazelwood, 2012). A multitude of subreceptors constitute a multitude of possible 5HT-related disorders. Elevated levels of 5HT is implicated in anxiety disorders, obsessive compulsive disorder and eating disorders, whereas low levels are associated with depressed mood (Iversen, 2006). Some serotonergically active medications are associated with treatment for migraines and extreme nausea. These disorders can be treated by increasing the amount of 5HT in the synaptic cleft by inhibiting reuptake mechanisms, or depleting the 5HT stores and essentially reducing its synthesis via a negative feedback mechanism or decreasing its action via receptor-downregulation (Iversen, 2006).

2.3.3.2 The noradrenergic system

NE is a direct effector of adrenergic cells, specifically at α and β receptors. These receptors are found in all major organ systems. Activation of these receptors results in increases in systolic, diastolic and pulse pressures, with little or no effect on cardiac output (Widmaaier *et al.*, 2006). Adrenergic receptors are prevalent in a variety of areas in the CNS, including the central nucleus of the amygdala, the limbic system and the hippocampus, all of which are brain areas affected in MDD (Molinoff, 2012). The amount of TH available in these regions determines the production of DA and NE. NE can also be converted to adrenaline (or epinephrine), a critical neurotransmitter used in situations of panic and stress (Sander-Bush & Hazelwood, 2012; Widmaaier *et al.*, 2006). In the cortex, TH is associated with adrenergic neurons and with dopaminergic neurons in the striatum (Murrin *et al.*, 2007). The NET is an important target for some antidepressants, indicating a role for NE in the development of depressive symptoms (Murrin *et al.*, 2007).

2.3.3.3 The dopaminergic system

Dopaminergic neurotransmission is directed by a family of two major receptor subtypes, D_1-like and D_2-like receptors, and its functions in the CNS includes memory, cognition, reward, emotion and motor activity (Sander-Bush & Hazelwood, 2012; Widmaaier *et al.*, 2006). Low concentrations in peripheral organs such as the kidneys, heart and vasculature play a role in blood pressure control through natriuresis, vasodilatation and cardiac contractility, while DA is also the primary regulator of prolactin secretion from the pituitary gland (Widmaaier *et al.*, 2006). Dysregulation of DA neurotransmission is implicated in Tourette's syndrome, Parkinson's disease, depressive disorders, schizophrenia, ADHD, bipolar disorder and substance abuse disorder (Andersen, 2003; Sander-Bush & Hazelwood, 2012). Disorders involving altered DA neurotransmission can be treated by inhibiting the DAT and thereby increasing the extracellular levels of DA (Iversen, 2006). Disorders involving pathological increases in DA,

such as schizophrenia, can be treated with DA receptor antagonists, preventing the activation of DA receptors (Sander-Bush & Hazelwood, 2012).

2.3.3.4 Memory and cognition

A very important function of DA in the hippocampus is to regulate certain processes of learning, memory and cognition. Recognition memory has multiple processes: acquisition, consolidation, retention, retrieval and performance, (Abel & Lattal, 2001). Object recognition memory as measured by the NOR, is a method of measuring declarative memory in rodents. Declarative memory is defined as the conscious memory of facts and events and can be categorised into personal (episodic) memory and general (semantic) memory (Winters *et al.*, 2008). This type of memory can be acquired by minimal encounters with the fact or event and DA receptor agonists or antagonists have the ability to influence the function of this type of memory (de Lima *et al.*, 2011). MA is documented to cause striatal dopaminergic and forebrain serotonergic neuron degeneration as well as somatosensory neurodegeneration in the cortical region of rodent brains (Marshall *et al.*, 2007). The perirhinal cortex, hippocampus and prefrontal cortex all form part of the cortical region of the brain that plays an important role in cognition (de Lima *et al.*, 2011; Winters *et al.*, 2008). MA-treated rats tested in the NOR for short- and long-term memory demonstrated no preference for the novel object, thereby showing an impairment in recognition memory (O'Dell *et al.*, 2011; Winters *et al.*, 2008).

2.3.4 Mechanisms of degeneration of neuronal systems

Numerous factors contribute to neurodegeneration and oxidative stress is very prominent among them (Andersen, 2004; Ischiropolous & Beckman, 2003). Many factors lead to the generation of reactive oxygen species (ROS) that are responsible for neuronal damage caused by oxidative stress. Infection, brain trauma, neurological disorders and exposure to neurotoxic substances can all activate immune system responses as a defence mechanism (Kreutzberg, 1996; Vilhardt, 2005). Glial cell activation, hyperthermia and ROS will be the focus of the neurodegenerative mechanisms presented in this section, whereas MA-specific mechanisms of neurodegeneration are discussed in section 2.4.3.

2.3.4.1 Astroglial and microglial activation

The non-neuronal cells of the brain are divided into macroglia and microglia. Macroglia are oligodendrocytes, astrocytes and ependymal cells and microglia are considered the macrophages of the brain (Leonard, 2003). Activation of glial cells accompanies pathological conditions of the CNS, such as ischemia, neurodegenerative disease, inflammation and trauma, and is described as a marker of neuronal injury (Kitamura *et al.*, 2010). Activated microglia have

phagocytic capabilities and play an important role in destroying foreign invading microorganisms, removing debris, promoting tissue repair by secreting growth factors and overall facilitating a return to homeostasis (Kreutzberg, 1996; Leonard, 2003; Widmaaier *et al.*, 2006). Astroglia have functions similar to microglia, including a role in CNS development, maintenance of ion homeostasis, neurotransmitter uptake and blood-brain barrier maintenance (Kitamura *et al.*, 2010). Microglia respond to changes in the brain's structural integrity and microenvironment but their role in neurodegeneration is not yet confirmed as neuroprotective or neurodegenerative (Graeber & Streit, 2010). They play a role in tissue repair after injury, much like phagocytes in peripheral organs but also possess cytotoxic capabilities. They are capable of releasing ROS (discussed in section 2.3.4.3), nitric oxide (NO), proteases, cytokines, arachidonic acid derivatives, excitatory amino acids (i.e. glutamate) and quinolinic acid, all of which are potentially cytotoxic substances in sufficient amounts (Kreutzberg, 1996; Graeber & Streit, 2010).

2.3.4.2 Hyperthermia

Compared to other organs, oxygen metabolism in the brain is significantly higher relative to its weight, producing a great amount of oxidative metabolism in the form of heat that needs to be regulated (Halliwell, 2006; Kiyatkin, 2005). The resulting hyperthermia resembles fever and can irreversibly alter the function of vital cellular components such as mitochondria and plasma membranes. Consequently, damaged cells can no longer function and are removed by phagocytic cells (Hildebrandt *et al.*, 2002; Kiyatkin, 2005; Lepock, 2003). Various drugs of abuse can impair thermoregulation and induce brain hyperthermia, exposing neuronal cells to a prolonged, elevated temperature. In fact, exposure to temperatures exceeding 40°C cause alterations in protein structure and function, ultimately leading to denaturation and neurodegeneration (Lepock, 2003). Dopaminergic neurons in the substantia nigra area are documented to be very temperature-sensitive, with raised temperatures resulting in an increase in firing activity (Kiyatkin, 2005). The substantia nigra is an important structure located in the midbrain, that plays an important role in learning, motor function and reward-motivated behaviour. Therefore, raised temperatures in the brain can alter the sensitivity and functionality of neurons that are responsible for important behavioural responses (Kiyatkin, 2005).

2.3.4.3 Reactive oxygen species

Cytosolic and extracellular non-enzymatic metabolism that involves oxidation leads to the formation of free radicals and reactive metabolites that are ultimately responsible for neuronal cell damage (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005; Thomas *et al.*, 2009).

Chemically reactive molecules containing oxygen, such as superoxide, hydroxyl radicals hydrogen peroxide and oxygen ions are termed ROS and elevated levels of these intermediates are associated with long-term damage to surrounding proteins and organelles (Meredith *et al.*, 2005; Thrash, 2010). Electron-deficient ROS binds with cysteinyl residues on important protein-structures and in so doing modifies protein structure and possibly function, with implications for energy production and amino acid metabolism (Halliwell, 2006). The oxidative species therefore react with other cellular substrates in its surroundings leading to oxidative damage (LaVoie & Hastings, 1999; Thrash, 2010), for example an attack on membrane lipids causes lipid peroxidation. Oxidative species are metabolised by endogenous antioxidant enzymes such as catalase, Cu/Zn superoxide dismutase and glutathione peroxidase (Krasnova & Cadet, 2009; LaVoie & Hastings, 1999). Elevated oxidative stress is now defined as a state where the greater amount of oxidative species exceeds the capacity of available anti-oxidant enzymes.

Peroxynitrite is a strong pro-oxidant byproduct formed following the oxidative metabolism of NO, a cellular signalling molecule and free radical (Halliwell, 2006; LaVoie & Hastings, 1999). Peroxynitrite is also able to react with the surrounding lipid membranes to cause lipid peroxidation by binding to cysteinyl residues. Nitrosylation is described as a post-translational protein modification process that is responsible for multiple types of cell-signalling and the process of nitrosylation is important in signal transduction where a NO group is attached to thiol group that forms part of the cysteinyl residues on proteins. (Marozkina & Gaston, 2012) When not controlled by anti-oxidant enzymes, the presence of ROS and reactive nitrogen species (RNS) such as peroxynitrite causes neurodegeneration (Krasnova & Cadet, 2009; Thrash *et al.*, 2010).

Glutamate is an excitatory amino acid and is capable of exciting virtually all neurons in the CNS, activating the ionotropic N-methyl-D-aspartate (NMDA) receptor (also present on all neurons) (Katzung, 2007). Excessive stimulation of the NMDA receptor also leads to the formation of ROS and RNS (Herleniuz & Lagercrantz, 2004) which is countered by various auto-regulatory mechanisms in the NMDA ion channel, including use-dependent receptor desensitization, protein kinase mediated phosphorylation and internalization (see Harvey & Shahid, 2012).

DA auto-oxidation (see section 2.4.3.2) produces a variation of oxidative metabolites, of which DA quinones are prominently cytotoxic. They are documented to bind to important molecules such as TH, DA neurons and DAT, consequently inactivating them (Kuhn *et al.*, 1999; Miyazaki *et al.*, 2006). Their cytotoxic activity contributes to the environment of oxidative stress and damage to neurons.

Several studies have demonstrated that a reduction of free ROS (via reduced production or enhanced clearance) is neuroprotective (Krasnova & Cadet, 2009; LaVoie & Hastings, 1999; Thrash, 2010). In this regard, genetically altered mice overexpressing the enzyme Cu/Zn-superoxide dismutase suffered less MA-induced neurotoxicity than controls (LaVoie & Hastings, 1999). For example, drug-induced oxidative stress in the rat brain is associated with increased lipid peroxidation, which can be reversed with the antioxidant, N-acetyl cysteine (NAC) (Harvey *et al.*, 2008). Importantly, NAC is also documented as an antidepressant in animal models (Ferreira *et al.*, 2008) and has been found to prevent social isolation induced changes in immune-inflammatory activity and associated changes in brain DA levels and behaviour (Möller *et al.*, 2013). Interestingly, NAC is effective for depressive symptoms in bipolar disorder (Berk *et al.*, 2008a) as well as for adjunctive treatment in schizophrenia (Berk *et al.*, 2008b).

2.4 Chemical properties and pharmacology of methamphetamine

2.4.1 Chemical and physical properties

MA is described chemically as a phenylethylamine amphetamine. It is structurally similar to the catecholamine neurotransmitters and acts as a powerful, indirect stimulant at endogenous monoamine receptors (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005; Nordahl *et al.*, 2003).

The full chemical name of MA is *n*-methyl-1-phenyl-propan-2-amine. MA exists as two enantiomers, viz. dextrorotary and levorotary stereoisomers. The dextrorotary isomer is considered to be the more biologically active optical isomer in the CNS than the levorotary isomer, which possesses more peripheral activity (Cruickshank & Dyer, 2009; Salocks & Kaley, 2003; Shep *et al.*, 2010). The most common chemical forms of MA include a liquid free base and a crystalline hydrochloride salt (MA-HCl), of which visual presentations can be seen in Figure 2-3. The latter is formed by bubbling gaseous hydrochloric acid through the free base (HSDB, 2005). MA-HCl is the more stable form and is identified as a white, odourless crystalline substance with a bitter taste. The free base is a clear, colourless liquid at room temperature, with a sharp biting odour resembling geranium leaves (HSDB, 2005; Salocks & Kaley, 2003).

Figure 2-3: Methamphetamine in crystallised form and freebase, respectfully (emcdda.europa.eu, 2013; sciencemadness.org, 2013).

MA-HCl is sensitive to light, is hygroscopic and soluble in water (1:2), alcohol (1:3) and ether (weakly). It is miscible with chloroform and very stable in an aqueous solution and can be kept in airtight containers at room temperature (Morris; 2007). A saturated solution in water is alkaline to litmus Even though the melting point is 171 ± 1°C , the salt will sublimate if heated slightly, which is the method used for smoking MA. The boiling point is 212°C at 760 mmHg (Cruickshank & Dyer, 2009; Salocks & Kaley, 2003).

The chemical structure of MA is similar to those of amphetamine and ephedrine and consequently it has comparable biological stimulant properties (Anglin *et al.*, 2000; HSDB, 2005). MA is more lipid-soluble than amphetamine and is readily absorbed through multiple administration routes. It is largely metabolised in the liver via *N*-demethylation, aromatic hydroxylation and ß-hydroxylation to produce amphetamine, 4-hydroxymethamphetamine and norephedrine, respectively (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005; Nordahl *et al.*, 2003). Furthermore, the structure of MA is very similar to that of DA (Figure 2-4), allowing uptake into DA axons (Iversen, 2006).

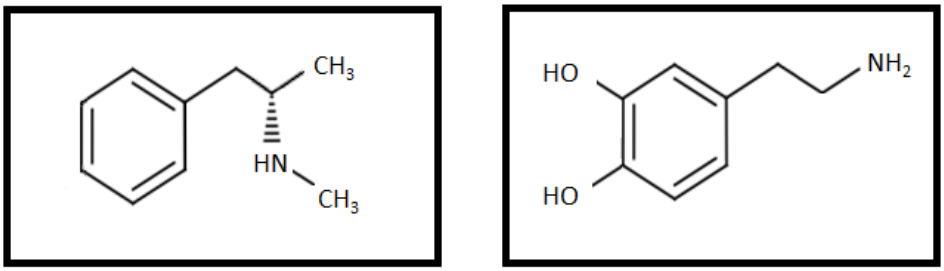

Figure 2-4: Comparison of the similarities between the structures of *d*-methamphetamine and dopamine (HSDB, 2005; medicinescomplete, 2012).

2.4.2 Neurological mechanisms of methamphetamine action

MA serves as a substrate for SERT, NET, DAT and VMAT-2. The DAT, SERT and NET are membrane-bound reuptake mechanisms for the reuptake of DA, 5HT and NE, respectively, from the synaptic cleft. The VMAT-2 is responsible for the packaging of these monoamines into cytosolic storage vesicles. On binding with the transport mechanisms, MA reverses their function (Nordahl *et al.*, 2003; Riddle *et al.*, 2006). Therefore, MA inhibits the reuptake of these neurotransmitters and reverses the flow of the content in the storage vesicles and neurons, leading to increased levels of the monoamines in the cytosol and the synaptic cleft (Cruickshank & Dyer, 2009; Krasnova & Cadet, 2009; Sulzer *et al.*, 2005). In particular, the acute administration of a high dose of MA increases the level of extracellular DA by displacing vesicular stores of DA through reversal of the VMAT-2, reverse transport of DA through the DAT and inhibition of DA metabolism by MAO (Cruickshank & Dyer, 2009; Nordahl *et al.*, 2003). In a study investigating drug effects in the mouse striatum, it was demonstrated that MA increases neural oxidative stress via a mechanism that is very similar to that of neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). However, MPTP, but not MA inhibits MAO activity (Thrash *et al.*, 2010). Indeed, in a parallel study, Strauss (2013) also demonstrated that MA exerts a pro-oxidative action in rat brain.

The recent discovery that MA acts as an agonist on the trace amine-associated receptor-1 (TAAR-1) has revealed additional mechanisms of action (Grandy, 2007), most notably since the TAAR-1 is associated with DA-regulation through modulation of the DAT (Xie & Miller, 2007). MA is structurally related to biogenic amines and can be classified as a trace-amine, which may bind to the TAAR-1 and thereby modulate DAT. Trace-amines such as β-phenylethylamine, *para*-hydroxyphenylethylamine, octopamine, synephrine and tryptamine are referred to as "false neurotransmitters" or "microamines" and are present in every invertebrate and vertebrate species examined to date. They are similar to biogenic amine neurotransmitters in terms of structure, metabolism and tissue distribution (Grandy, 2007; Wainscott *et al.*, 2007). Importantly, trace-amines as well as biogenic amines and amphetamine-like substances activate G protein-coupled TAAR-1s (Xie & Miller, 2007). TAAR-1 is co- expressed with the DAT in a subset of DA neurons. When considering the array of possible agonists, it is likely to be involved in the pathology of psychiatric disorders that are associated with DA dysregulation, such as schizophrenia and ADHD. The TAAR-1 has also been implicated in the mechanism whereby amphetamine-like substances affect general metabolism and thermoregulation, cardiovascular homeostasis, olfaction and behaviour (Lindemann *et al.*, 2008; Reese *et al.*, 2007).

2.4.3 Neurobiology of MA-mediated neurotoxicity

Most cases of drug-induced neurotoxicity arise from chronic drug abuse (Büttner, 2011; Renoir *et al.*, 2012). During adolescence the structure and function of the brain undergoes dramatic rearrangement and thereby adolescence represents a turning point in how the brain adapts to external influences (Andersen, 2003). The adult brain is considered to be less vulnerable to external influences, but retains marginal ability to change its neurochemical function, thereby to adapt to the challenges it has been presented with (Henn & Vollmayr, 2004; Parent, 2003).

Neurotoxicity may result from direct pharmacological effects of the substance itself, or when the substance stimulates the formation of toxins via altered metabolic routes (LaVoie & Hastings, 1999; Miyazaki *et al.*, 2006). A metabolic cascade of events following MA administration is evident in recent literature, involving glial cell activation, formation of ROS, hyperthermia, reactions with NMDA receptors, mitochondrial dysfunction and malfunction of monoamine reuptake mechanisms and monoamine metabolism. These represent the mechanisms by which MA exposure can damage the neuronal system (Thomas *et al.*, 2008; Thrash *et al.*, 2010; Nordahl *et al.*, 2003). All of these mechanisms may ultimately lead to axonal damage or neuronal loss, and/or hypoperfusion due to altered arterial contractility (Figure 2-5) (Büttner, 2011; Frost & Cadet, 2000; Krasnova & Cadet, 2009; Thomas *et al.*, 2004). The degree of recovery of monoaminergic function following MA exposure has not been established, although significant improvements of anxiety and depressive symptoms are seen in patients abstaining for an average of 3 weeks (Meredith *et al.*, 2005).

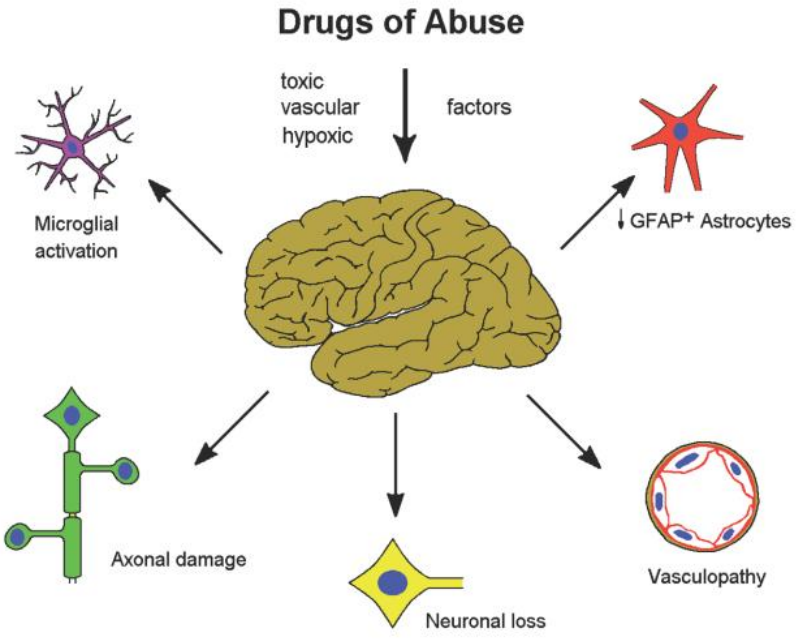

Figure 2-5: Damage to neuronal components as a result of drug abuse (Büttner, 2011).

Following MA administration, the subsequent chain of neurobiological events described in Figure 2-5 culminates in oxidative stress and axon- and neurodegeneration. Mechanisms of oxidative neurotoxicity are significantly implicated in the neurodegenerative properties of MA (Cruickshank & Dyer, 2009; Nordahl *et al.*, 2003; Thrash *et al.*, 2010). Regenerative capability of the brain ensures its recovery after an acute, neurotoxic dose of MA, whereas chronic administration, even at low doses may result in long-lasting damage (Barr *et al.*, 2006; Cruickshank & Dyer, 2009; Glasner-Edwards *et al.*, 2008; Murrin *et al.*, 2007). Neurotoxicity, both as a result of chronic and acute MA administration, is therefore a result of a variety of mechanisms that can leave both temporary and lasting neurochemical and structural changes in the brain.

2.4.3.1 Oxidative stress

Upon administration MA generates a significant amount of ROS via several mechanisms, including glial cell activation, auto-oxidation of DA in the synaptic cleft (discussed in the following section), prolonged increases in glutamate release, and increased metabolism of NO (Krasnova & Cadet, 2009; Zhang *et al.*, 2009). These are known to have a negative impact on neuroplasticity. In addition, concurrent MA-induced hyperthermia denaturates proteins, adding to the damaging effects of ROS (Kiyatkin, 2005).

As discussed in section 2.2.2, the glial cells of the CNS are capable of releasing cytotoxic substances that, in turn, cause oxidative stress. Marked glial cell activation have been demonstrated to result from different neurotoxic regimes of MA in animals, relative to untreated controls (LaVoie *et al.*, 2004; Thomas *et al.*, 2008; Yamamoto *et al.*, 2010). MA administration accompanies activation of both microglia and astroglia, so that these cell types have been implicated as mediators of MA-induced neurotoxicity (Kitamura *et al.*, 2010). Astroglial cells normally suppress microglial activation through the release of anti-inflammatory cytokines and neurotrophic factors. Astroglia also release glutamate which in turn cause microglial activation. It is therefore uncertain under which conditions the respective glial cells mediate an inflammatory response to MA (Yamamoto *et al.*, 2010).

Marked and prolonged increases in glutamate release have been documented with acute MA administration (Nordahl *et al.*, 2003). Glutamate is an excitatory amino acid and is capable of exciting virtually all neurons in the CNS, activating the ionotropic NMDA receptor which is also present on all neurons (Katzung, 2007). Excessive stimulation of the NMDA receptors is also known to promote the formation of ROS, which may in part explain the role of oxidative stress in MA-induced neurotoxicity (Herleniuz & Lagercrantz, 2004). Post-mortem studies of known

MA abusers revealed conflicting data to that of experimental animals receiving chronic MA (Kitamura *et al.*, 2010). The proliferation of activated glial cells in areas with prominent dopaminergic nerve terminal damage is observed in the animals but not in the human participants. This may be attributable to the regenerative ability of the neurons in humans (Parent, 2003). In addition, human data are complicated by the fact that they are obtained post mortem with the frequency and dosage of the MA use prior to death usually unknown (Frost & Cadet, 2000). No records of MA use prior to the post-mortem studies were kept and therefore a reliable conclusion in this regard cannot be made.

2.4.3.2 DA auto-oxidation

MA has been shown to inhibit the neuronal reuptake and enzymatic metabolism of DA, resulting in elevated DA levels. Both extracellular and cytosolic DA are exposed to non-enzymatic metabolism involving oxidation, hence leading to the formation of free radicals and reactive metabolites which are ultimately responsible for neuronal cell damage (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005; Thomas *et al.*, 2009). The prolonged presence of monoamines in the neuron cytosol and in the synaptic cleft results in their non-enzymatic degradation and the formation of neurotoxic metabolites, most notably the toxic metabolites of DA and 5HT (6-hydroxydopamine and 5,6-dihidroxytryptamine respectively) as well as DA quinones (Napolitano *et al.*, 1995). All of these monoaminergic metabolites are associated with oxidative stress, thereby relating both DA and 5HT with MA-induced neurotoxicity (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005; Riddle *et al.*, 2006).

Figure 2-6 shows a schematic representation of the metabolic cascade of DA oxidation promoting the generation of ROS that are implicated in the neurotoxic degeneration of DA axon terminals (Meredith *et al.*, 2005; Napolitano *et al.*, 1995). DA quinones bind with protein cysteinyl residues to form cysteinyl-DA, a stable oxidative metabolite of DA (LaVoie & Hastings, 1999). Once cysteinyl-DA reacts with the surrounding proteins and vital cellular structures such as mitochondria, it causes dysfunction and protein-inactivation, which may lead to apoptosis (Thrash, 2010; LaVoie & Hastings, 1999). Additionally, cysteinyl-DA is capable of reacting with, and inactivating, important anti-oxidants such as glutathione, further promoting a pro-oxidative environment culminating in oxidative stress (Miyazaki *et al.*, 2006). DA quinones are also powerful activators of microglia, which mediate an inflammatory response (Yamamoto *et al.*, 2010). Therefore it can be envisioned that, although DA may be necessary for MA-induced neurotoxicity by producing ROS and RNS, microglial activation also provides a pro-

inflammatory stimulus whereby it contributes to eventual neurodegeneration (Sekine *et al.*, 2008; Yamamoto *et al.*, 2010).

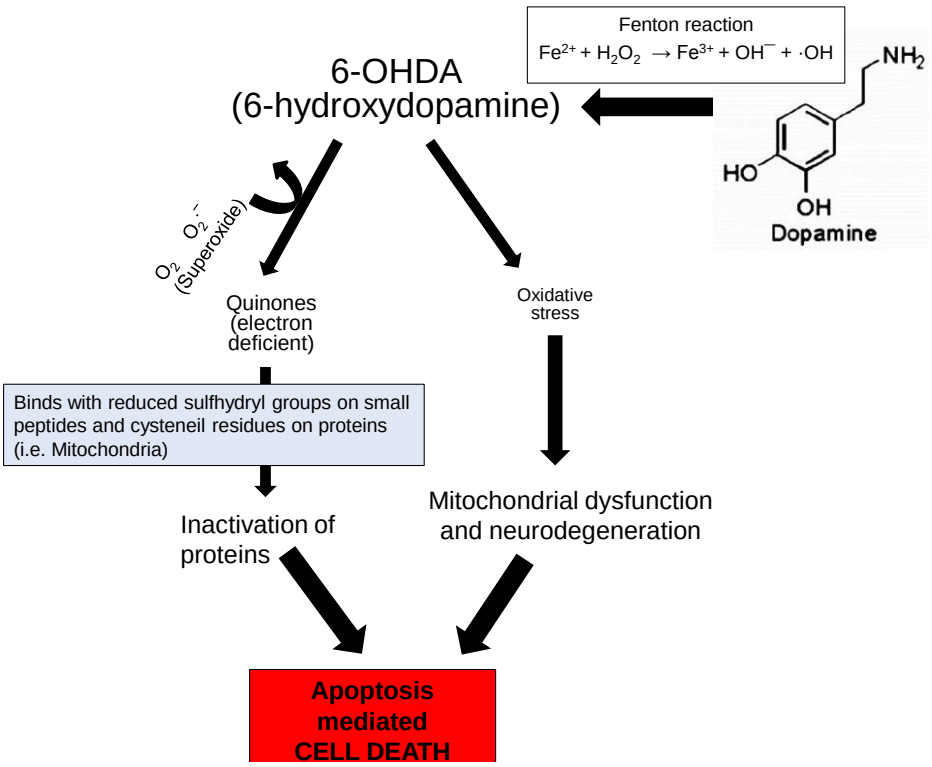

Figure 2-6: Metabolic cascade of non-enzymatic DA oxidation, its interaction with proteins and mitochondria, and how this may lead to apoptosis.

Recent data has suggested and then confirmed that MA toxicity is partly dependent on the presence of endogenous DA (Krasnova & Cadet, 2009; Meredith *et al.*, 2005; Thomas *et al.*, 2009). This is illustrated by pre-treatment with α-methyl-*p*-tyrosine (AMPT), an inhibitor of TH, which prevents the synthesis of DA and thereby exerts neuroprotective effects by preventing MA-induced damage to dopaminergic and serotonergic neuron terminals. This phenomenon is confirmed by the observation that the administration of L-DOPA exacerbates MA-induced toxicity. However, pre-treatment with reserpine, which depletes the available stores of DA by disrupting storage vesicles, exacerbates neurotoxicity. MA causes DA expulsion into the synaptic cleft and pre-treatment with reserpine results in greater neurotoxicity when compared to pre-treatment with AMPT. Reserpine leaves the synthesising capacity intact whereas AMPT prevents it, thereby relating the cause of neurotoxicity to newly synthesised DA pools. In conclusion, the elevation of synaptic DA levels exacerbates, and the reduction of DA levels reduces MA-induced neurotoxicity (Nordahl *et al.*, 2003; Thomas *et al.*, 2009).

2.4.4 Methamphetamine-induced psychiatric complications

In addition to the altered monoaminergic function caused by MA exposure, other aspects of monoamine metabolism are also affected. In the cortex, TH is associated with adrenergic neurons and in the striatum, with dopaminergic neurons, thereby differentially affecting behaviour in these brain areas (Murrin *et al.*, 2007). TH is the rate-limiting enzyme for the synthesis of DA and NE and disruptions in neurotransmission of these monoamines (for example via altered TH-mediated synthesis) can elicit serious behavioural deficits (López-Muñoz & Alamo, 2009). Post mortem studies have revealed decreases in striatal TH enzyme activity in MA users, while controlled animal studies have supported these findings (Krasnova & Cadet, 2009; LaVoie & Hastings, 1999; Meredith *et al.*, 2005; Nordahl *et al.*, 2003). Amphetamines increase the activity and expression of TH and consequently the synthesis of DA and NE (Barr *et al.*, 2006). MA, however, inhibits TH activity, possibly due to an inhibition mechanism elicited by an elevated extracellular concentration of DA or a subsequent downregulation of TH (Sulzer *et al.*, 2005). At high doses MA reversibly decreases the activity of tryptophan hydroxylase (TrH) activity, the rate-limiting enzyme for the synthesis of 5HT. Eventually the repeated administration of MA causes long-lasting damage to monoaminergic neurons which could ultimately lead to depressive behaviour (Fleckenstein *et al.*, 1997).

2.4.4.1 The role of methamphetamine in psychiatric disorders

Whereas a genetic predisposition to develop psychiatric disorders (such as MDD or schizophrenia) is well-recognised, drug abuse, such as with MA addiction might also play an important role in the development of these disorders (Andersen, 2003; Forty *et al.*, 2008; Hasler *et al.*, 2004). In fact, MA addiction may exacerbate depressive behaviour by significantly altering monoaminergic metabolism and function. MA is documented to cause metabolic and structural changes in the striatum of chronic MA abusers (London *et al.*, 2004). Such changes are also characteristic of certain neuropsychiatric disorders, such as striatal abnormalities which have been shown to be associated with the neuropathology of schizophrenia (Matheson *et al.*, 2011; Möller *et al.*, 2013), and smaller hippocampal volumes and impaired memory which are associated with MDD (Chang *et al.*, 2007; Sapolsky *et al.*, 2000). Drug induced changes in neuronal function are more prominent during neurodevelopmental phases, such as with prenatal MA exposure in humans, which has been shown to have qualitatively similar, yet quantitatively more pronounced neuropsychological outcomes than following exposure during adulthood (Andersen, 2003). This may suggest that the developing brain is more vulnerable to the influences of MA than a mature brain (Chang *et al.*, 2007).

The neuropathology of drug abuse and of MDD has been discussed earlier (sections 2.1.3 & 2.1.4). It should be noted that MDD is the one of the most prevalent psychiatric complications of drug addiction, depending on frequency and dosage of drug use (Büttner, 2011; Zweben *et al.*, 2004). Hence, exposure to centrally active substances during development puts the subject at greater risk of developing a psychiatric disorder such as MDD, schizophrenia, Tourette's syndrome or ADHD (Andersen, 2003). Additionally, MA abuse has the ability to increase the risk to develop Parkinson's disease (Thrash *et al.*, 2009). The focus on psychiatric disorders, however, takes precedence when considering the high treatment demand for individuals suffering MA-induced psychoses and –depressive symptoms (Vos *et al.*, 2010; Zweben *et al.*, 2004).

Depressed mood, irritability and suicidal ideation have been reported in patients experiencing MA withdrawal as well as during active MA use (Büttner, 2011; Meredith *et al.*, 2005; Zweben *et al.*, 2004). Importantly, the frequency and degree of MA abuse along with the route of administration are positively linked to withdrawal symptoms, which can predict the occurrence of a mood disorder (Anglin *et al.*, 2000; Domier *et al.*, 2000; Meredith *et al.*, 2005).

Psychosis is one of the many CNS effects caused by MA abuse. MA abuse may also potentially exacerbate symptoms of existing schizophrenia, anxiety-related disorders and MDD (Darke *et al.*, 2008). MA-induced increases in DA content have been documented to produce hallucinations and delusions similar to those seen in schizophrenia (Cruickshank & Dyer, 2009; Darke *et al.*, 2008). Prevalence of MA-related psychotic episodes outranks that of the general population, as well as psychoses seen in individuals who abuse cocaine on a regular basis (Glasner-Edwards *et al.*, 2008). A MA psychosis is defined as a MA-induced hallucinatory or paranoid state that cannot be distinguished symptomatically from acute paranoid schizophrenia (Cruickshank & Dyer, 2009; Nordahl *et al.*, 2003). Hallucinations can manifest in all senses, with auditory and visual hallucinations as the most common (Darke *et al.*, 2008). Tactile hallucinations are also common, manifesting as hallucinations of insects crawling under the user's skin which leads to skin-picking and compulsive scratching (Nordahl, *et al.*, 2003). Symptoms of psychoses can last hours and even days and severe cases may require sedation and antipsychotic medication (Meredith *et al.*, 2005).

Treatment of MA-induced psychoses and depressive symptoms are currently treated similar to that of schizophrenia and depression, utilising either antipsychotics, antidepressants or drug rehabilitation as monotherapy or in combination (Kay-Lambkin, 2008; Meredith *et al.*, 2005).

2.4.5 Methampetamine-induced teratogenicity

2.4.5.1 Drug abuse during pregnancy

Pharmacological sensitivity of the CNS changes during the phases of neurodevelopment, so that the vulnerability of the CNS neurocircuitry to neurotoxic substances is also dependent on the neurodevelopmental phase. Different brain regions and neurotransmitter systems develop asynchronously, suggesting that the same intervention at different stages of neurodevelopment will affect different regions or transmitter-systems, thereby resulting in different outcomes of neurodevelopment (Andersen, 2003; Murrin *et al.*, 2007). It follows logically that the resulting differences in the mature nervous system will be associated with corresponding subtle to marked differences in the response to a pharmacological agent. Figure 2-7 illustrates one model of how drug challenges during different neurodevelopmental phases may result in differential (even opposite) outcomes (Acuff-Smith *et al.*, 1996; Andersen, 2003). According to this model, drug exposure during the early postnatal developmental phase (juvenile drug exposure) elicits adaptation and tolerance, resulting in a reduced response upon re-exposure in adulthood. To the contrary, exposure in puberty results in sensitisation and hence an exacerbated response following re-exposure in adulthood. This implies that prenatal exposure to pharmacological agents may significantly affect neurodevelopment and that early-life exposure may also affect pharmacology later in life when the neural system has fully matured (Andersen, 2003).

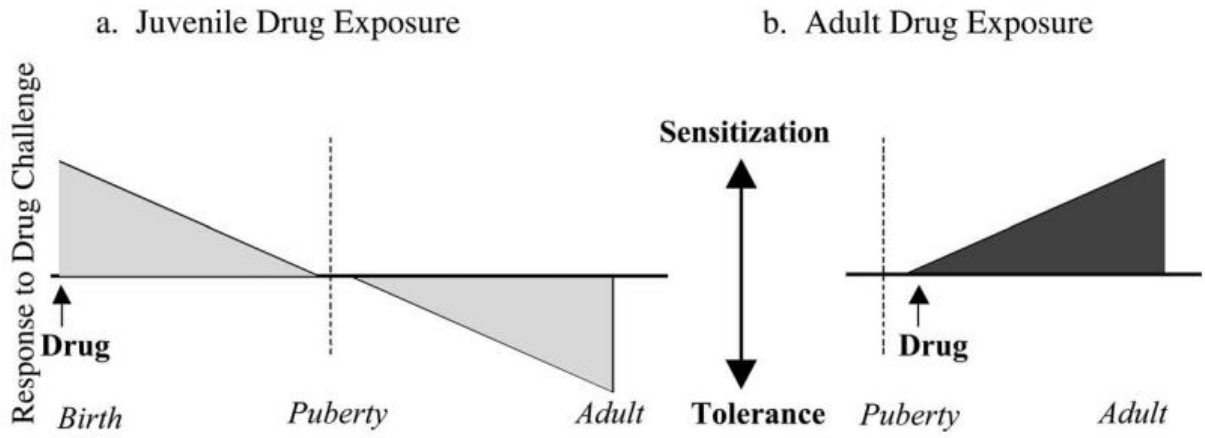

Figure 2-7: Diagram of the different responses to drug challenges in adulthood, depending on previous drug exposure during either the prenatal phase or in puberty (Andersen, 2003).

Figure 2-8 shows the age distribution of primary MA users between 2004 and 2006. Importantly, the majority of MA users are adolescents and young adults, stages when neurodevelopment is not fully mature yet. Considering the biological development (albeit hormonal, behavioural and neuronal) still to take place in this population, the effect of MA-

related disease and dysfunction raises fundamental concerns. Furthermore, many of these individuals are sexually active, increasing also the risk of pregnancy and thereby the risk of prenatal exposure of unborn babies. The demand for this drug on the South African black market (particularly the Western Cape Province) was found to peak around 2004, at which time it was documented to be the fastest growing abuse of any drug in the country (Plüddeman *et al.*, 2008). When assessed in children aged 3-16 years, prenatal exposure to MA has been found to result in a smaller putamen, a smaller globus pallidus (left and right) and smaller hippocampal volumes than in controls not exposed (Chang *et al.*, 2004). These brain structures correspond with those structures affected in adult MA addicts, albeit to a greater magnitude. Both adults and children, however, had a similar reduction in hippocampal volumes (Chang *et al.*, 2007). These data again underlines the notion that the developing brain responds differently to MA exposure so that adolescent MA abusers, or children exposed to MA prenatally are considered to be more vulnerable to late-life effects on brain function and behaviour.

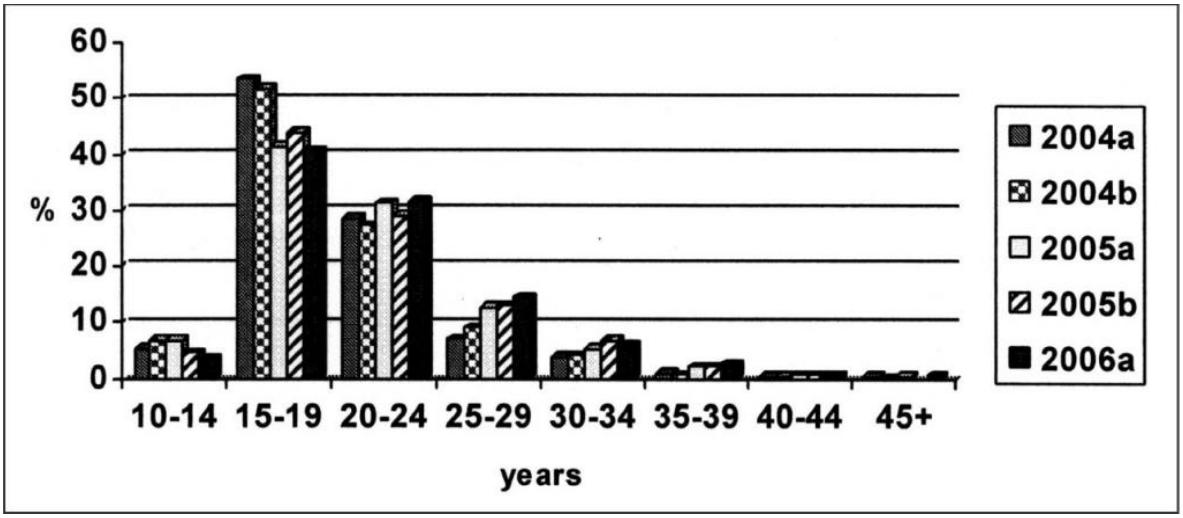

Figure 2-8: Graph of age distribution among primary MA users between 2004 and 2006 in treatment centres in Cape Town, South Africa (Kenneth & Geerts, 2007).

2.4.5.2 Methamphetamine abuse and teratogenicity

One of many serious problems caused by the abuse of MA is birth-defects in new-born babies as a consequence of MA abuse by pregnant mothers. Because of the power of addiction, women who fall pregnant are not likely to quit the use of the drug for an extended period of time to allow the development of a healthy baby. A recent on-going study in Cape Town, however, reported that two thirds of pregnant women in the cohort reported decreased MA use during pregnancy which begs an explanation (Madide *et al.*, 2012). The reason for this is not clear, however. Of note is that MA use is decreased and not abstained from. Dangers of drug abuse, however, are not limited to the effect of the substance on the mother or the child. Transmission

of HIV, sexually transmitted disease and the presence of viral hepatitis are also concerns of the health of the mother and her unborn child (Madide *et al.*, 2012).

Many MA addicts are multi-drug users, making it difficult to isolate the effects caused solely by MA (Kenneth & Geerts, 2007). Case studies report erratic attendance, late enrolment for antenatal care or no antenatal care at all but yield data with insufficient statistical power to make any conclusive findings in this regard (Kenneth & Geerts, 2007; Madide *et al.*, 2012). Due to the lack of appropriate clinical data on the effect of prenatal exposure of MA in humans, our current understanding thereof is limited to findings provided by animal studies (Acuff-Smith *et al.*, 1996; LaGasse *et al.*, 2011; Madide *et al.*, 2012; Williams *et al.*, 2002). Data from animal studies demonstrate that prenatal exposure to MA alters the neurodevelopment of the rat foetus, consequently with long-lasting or irreversible effects (Bubenikova-Valesova *et al.*, 2009; Smith *et al.*, 2008; Vorhees *et al.*, 2000; Won *et al.*, 2001). The effect of MA on developing neurocircuitry has been documented both in studies with animal models as well as follow-up studies in women abusing MA during their pregnancy (Madide *et al.*, 2012).

Untoward effects associated with MA exposure/abuse during gestation can be potentiated by concurrent nicotine and alcohol abuse and include perinatal mortality, intraventricular haemorrhage, foetal growth restriction, increased risk of preterm labour, placental abruption, decreased birth weight, cardiac defects, cleft palate and behavioural effects in neonates (Cruickshank & Dyer, 2009; Meredith *et al.*, 2005; Won *et al.*, 2001). Low rates of prenatal care, pre-eclampsia, unplanned caesarean, increased susceptibility to neonatal infection and maternal anaemia have also been recorded in cases of MA abuse during pregnancy (Cruickshank & Dyer, 2009). Even postnatally, when the child is not directly exposed, MA abuse by caretakers may negatively affect the psychosocial development of the child by contributing to the development of a socioeconomical environment that promotes the abuse and neglect of young children (Anglin *et al.*, 2000; Kenneth & Geerts, 2007).

Animal studies investigating prenatal MA exposure have shown a significant decrease in birth weight, length and head circumference, as well as the malformation or other abnormalities of the extremities, cardiovascular system, CNS, and intestinal and urogenital systems (Kenneth & Geerts, 2007; Won *et al.*, 2001). The developmental phase of exposure is also important. For example, delayed acquisition of locomotor abilities were reported in pups that received prenatal MA during early gestational days 7-12, whereas exposure during late gestational days 13-18 did not seem to affect this ability (Acuff-Smith *et al.*, 1996). Furthermore, following pre-natal exposure to MA, eye defects in new-born rat pups have been reported in several studies (Acuff-

Smith *et al.*, 1996; Won *et al.*, 2001). Vorhees and colleagues confirmed that exposure to MA on postnatal days 11-20 leads to spatial navigation and memory deficits, a function of the hippocampus (Vorhees *et al.*, 2000).

2.4.6 Theoretical framework for long-lasting behavioural deficits

A healthy brain and body have excellent regenerative capabilities and the reason for the length of enduring deficits in behaviour and brain chemistry caused by drug exposure is therefore questioned. As discussed in section 2.4.5.1, still-developing neural networks are vulnerable to external influences such as drugs, specifically during critical stages in development. These stages are described as periods of rapid change when neurotransmitters and modulators stimulate processes into action for correct growth, development and integration of neural networks (Andersen, 2003; Herleniuz & Lagercrantz, 2004). Critical stages in neurodevelopment occur throughout the developmental period up to the end of puberty, a particularly important event being the overproduction and pruning of synapses (Andersen, 2003). An overproduction of synapses accompanies major developmental transitions such as birth, childhood, adolescence and puberty and pruning is a term that refers to the elimination of synapses to levels of the mature brain. This important process precedes and often marks the transitional phases of development (Andersen, 2003; Herlenius & Lagercrantz, 2004).

Stressful occurrences such as drug exposure or severe emotional trauma during these critical stages can cause greater or longer-lasting damage than during maturity. MA exposure during early development is discussed in section 2.4.5. Early-life adversity in the form of emotional stress can manifest in several environmental factors, such as poverty, ineffective parenting methods, parental disharmony and separation, an antisocial family and attention or learning-deficit (Farrington, 1993), contributing to social dysfunction and emotional stress. Singular traumatic events such as the death of a family member or sexual abuse cause even greater amounts of emotional stress, leading to various hormonal and neurochemical changes (Swaab *et al.*, 2005). The importance of social dysfunction and emotional trauma during the early years of development is underemphasised in their role to contribute to adaptive behavioural deficits that can lead to anxiety- and depressive-like behaviour.

Dynamics of adaptive behaviour and neural development have also been hypothesised to be selectionist or constructivist in its mechanism. The selectionist theory is also referred to as neural Darwinism, a concept first proposed by Gerald M. Edelman in 1978 (described by Seth & Baars, 2005), which suggested that a "selection" of synapses to develop occur in response to the biological feedback of events, and is the mechanism by which neurodevelopment alters to adapt

to the environment (McDowell, 2010; Quartz & Sejnowski, 1997; Seth & Baars, 2005). The constructivist theory suggests that neural development can be directed by its environment and is 'instructed' to develop in a specific way to match the needs of the environment (Quartz & Sejnowski, 1997). In short, behaviour is selected by its consequences (neural Darwinism) or events select behaviour (constructivism).

Neurodevelopment (as discussed in section 2.3.3) can be influenced by many factors but when critical developmental periods are affected, lasting alterations to the structure and function of neural processes are very likely.

2.5 Summary and conclusion

MA is an easily accessible, yet powerful and addictive psychostimulant. Moreover, it is capable of eliciting depressive-like behaviour and psychosis in abusers, and has also been shown to significantly influence neural networks in the brain by affecting neuronal metabolism and even brain structure. The exceptionally fast growing nature of this social problem has created an urgency to better understand the mechanisms underlying its destructive potential and to explore ways to treat and combat its abuse.

MDD is associated with drug abuse and particularly stimulant abuse, creating a need for knowledge on the long-term consequences of MA abuse. Additionally, *in utero* exposure can predispose an individual to develop psychiatric disorders such as MDD later in life. Also, whereas the neuropathology of MDD is yet to be fully understood, understanding the role of MA abuse in the development of MDD can provide additional insight into this question.

Relatively little is known about the teratogenic effects of MA as is clear from clinical observations. In particular we need to better understand the differences in the effects of juvenile and adult exposure to MA. We also need to understand the preventability and reversibility of its consequences, as well as the role of genetic prevalence in the manifestation of neuropsychological disorders following MA exposure. The development of a suitable animal model to study these phenomena will assist greatly in our endeavours to understand and treat the consequences of MA abuse.

2.6 General commentary

The focus of the current study lies on three major areas of interest: the neurobiology of MDD, the influences of MA on psychiatry and mechanisms of the teratogenic influences of MA on neurodevelopment. Consequently, the literature review provided a broad view of the three areas.

A schematic diagram is provided to illustrate the perceived relationship between these areas (Figure 2-9).

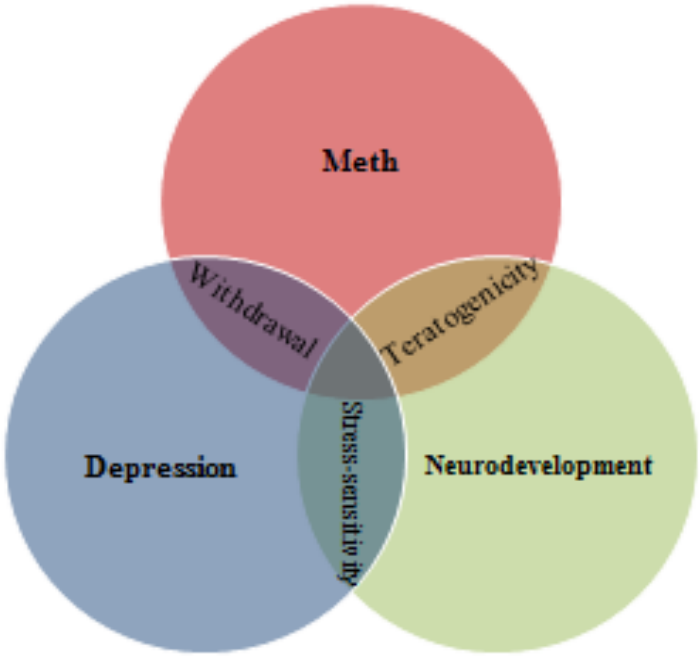

Figure 2-9: Schematic diagram representing the complicated relationship of MA, depressive behaviour and neurodevelopment.

3 Chapter 3 Materials and methods

This Chapter provides a detailed explanation of the experimental layout, experimental methods, materials, animal models, drug usage and behavioural tests that were employed in the current study. The current study has been approved by the Research Ethics Committee of the North-West University (ethics approval number NWU--000105-11-S5) and was performed in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

3.1 Overview of the study layout

The current study employed FSL rats, a stress-sensitive rat line, and FRL rats as their controls. For the current study, animals were treated with saline (vehicle control) or MA during one of four age-related phases (viz. phases early in life), namely prenatal, postnatal, prepuberty or puberty, as seen in Table 3-1.

The age groups were measured in natal days (ND), for example, gestational (prenatal) days are negative natal days and postnatal days are positive. Three of the age groups received either a subcutaneous (s.c.) MA or saline injection twice daily for 16 days, and were housed normally until behavioural assessments. The prenatal age group received a fixed-dose administration once daily for 16 days and was housed normally until offspring reached ND+60. Behavioural assessment commenced on ND+60 for all treated rats and the offspring of the treated dams. Table 3-1 also explains the escalating dose range used for each age group.

Table 3-1: MA and vehicle treatment groups according to age (ND = natal days).

Group	Administration days	Dosage range
Prenatal	ND-13 to ND+2	5 mg/kg injected s.c. daily for 16 days
Postnatal	ND+3 to ND+18	0.2 - 6.0 mg/kg increased with 0.2 mg/kg per s.c. administration twice daily
Prepuberty	ND+19 to ND+34	0.2 - 6.0 mg/kg increased with 0.2 mg/kg per s.c. administration twice daily
Puberty	ND+35 to ND+50	0.2 – 6.0 mg/kg increased with 0.2 mg/kg per s.c. administration twice daily
Behavioural tests occur on ND+60 for all male rats		

A schematic representation of the age-specific treatment groups is presented in Figure 3-1. The 10 day gap between treatment of the last age group and behavioural assessment ensures

sufficient wash-out effect of the drug, thereby ruling out any residual direct effect of MA on the test results.

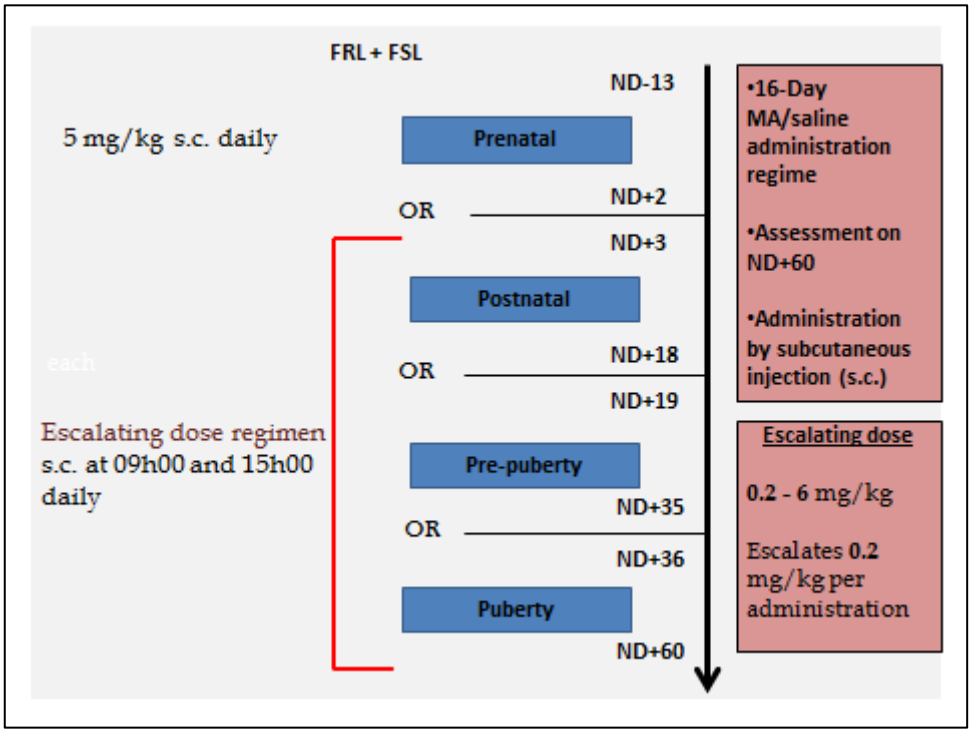

Figure 3-1: Schematic representation of treatment groups regarding drug treatment (FRL = Flinders Resistant Line rats; FSL = Flinders Sensitive Line Rats; MA = methamphetamine; ND = natal day).

3.2 Animals and materials

Behavioural test apparatus for the forced swim test (FST), the open field test (OFT) and the novel object recognition test (NOR) is discussed in each respective section of the behavioural test discussions below. Apparatus for these tests were custom-designed and manufactured by the Instrument Makers at the Potchefstroom Campus of the North-West University, according to specifications published and validated in our laboratory. Digital video cameras were mounted over or opposite the behavioural test apparatus to record proceedings and were operated by Leiosoft Video Capture® software.

3.2.1 Drug usage

Legal permits to respectively import and possess MA were obtained from the Department of Health of South Africa.

- Import-permit for narcotic drugs and/or psychotropic substances: IMP/626/2011
- Permit to acquire, possess and use schedule 5 and 6 substances for the purpose of education: POS107/2011/2012

Thereafter MA-HCl was purchased from Sigma Aldrich, Missouri, USA. The MA was dissolved in isotonic saline purchased at Kampus Pharmacy, Potchefstroom as vehicle, with the vehicle control containing saline without the drug. Chemical properties of MA-HCl are discussed in Chapter 2, section 2.4.1.

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

Animal models can be used to study the biobehavioural aspects of corresponding human disorders. Whenever the objective is to improve our understanding of anything that cannot be studied under controlled conditions, an experimental model can be introduced as a representative, standardised set-up or system, with the purpose to mimic responses to those conditions affecting the phenomenon under investigation. In context of an experimental animal model of disease, this can be defined as an animal with traits that are validated to represent aspects of the human condition, with the understanding that the response of this animal model to interventions will be comparable to the response of its human counterparts following the same intervention (Yadid *et al.*, 2000). Therefore, the objective of an animal model as surrogate for a psychological disorder in humans is to replicate as close as possible the human disorder (or aspects thereof) under controlled experimental conditions.

Animal models are assessed and validated according to major validation criteria, including face, predictive and construct validity and additionally, aetiological validity (McKinney & Bunney, 1969). These criteria have been refined over time and require that an animal model must resemble the human condition it is modelling in terms of observable traits and symptoms (face validity), treatment response (predictive validity), underlying neuropathology (construct validity) and corresponding causative factors of the disease (aetiological validity).

The current study utilised male FSL rats as the stress-sensitive model and the FRL rats as the control, bred in captivity in both the North-West University Animal Centre and the NHLS laboratory. The FSL rats are a genetic (inbred) rodent model of MDD and known to be inherently stress-sensitive, as opposed to their controls, the FRL rats (Overstreet, 1993; Overstreet *et al.*, 2005). As discussed in Chapter 2, the validity of the FSL rat model for representing MDD in humans is well documented. The face, predictive and construct validity of the FSL rat as an animal model for depression is discussed in Chapter 2, section 2.2.4. Inherent depressive-like behaviour of FSL rats can be seen in the FST, where they exhibit significantly increased immobility behaviour in comparison with FRL rats. Whereas the FST classically requires a 15 min conditioning swim session 24 h prior to the 5 min test swim session, FSL rats

do not require this pre-conditioning swim and can be assessed without it. Immobility in the FST relates to "helplessness", in which the rat moves only to keep its head above water, without struggling to escape. FSL and FRL rats are discussed in more detail in Chapter 2, section 2.2.5.

MDD shows a high rate of comorbidity with anxiety- and immunological disorders. However, FSL rats do not exhibit general anxiety compared to FRL rats, as evaluated in the elevated plus maze, but do display anxiety generated by peers, as measured in the social interaction test (Overstreet *et al.*, 2005). Furthermore, a decrease in specific immune function is noted in FSL rats, rendering FSL rats more susceptible to type 1 immunological deficit and adding to the construct validity of the model.

3.2.3 General housing protocol

All rats were housed in cages containing sawdust (bedding material) and were provided food and water *ad libitum*. The room was kept at a controlled temperature of 23°C ± 1°C, humidity (50%) and a 12 h light-dark cycle (switch occurred at 06h00 and 18h00).

For each treatment group a female FSL and FRL rat of breeding age were paired with their male genetic equivalent for two nights. On the morning of the third day the male rat was removed from the cage and the female was assumed to be gravid. This was marked as ND-21, relative to birth at ND0. At birth the female rat pups were removed from the litter, keeping only the male pups for further study. A maximum of 6 pups were kept per mother per cage until weaning on ND+21. Thereafter pups were housed normally until ND+60 when behavioural assessments are conducted.

Drug treatments were employed during the time of housing described above, with the administration methods described in section 3.3.1.

3.3 Methods

3.3.1 Drug administration and dosage

MA was stored away from light in a locked safe within a temperature controlled room, keeping a schedule 6 logbook of usage under the supervision of a registered pharmacist.

MA is a lipophilic drug that is easily absorbed into the bloodstream after s.c. injection. In general s.c. administration allows for accurate dosing, while posing less risk for injury than intraperitoneal administration, particularly when administering drugs to small pups for a length of 16 days. Four age groups (as mentioned in Table 3-1) were identified and presented in Figure 3-2 and defined as:

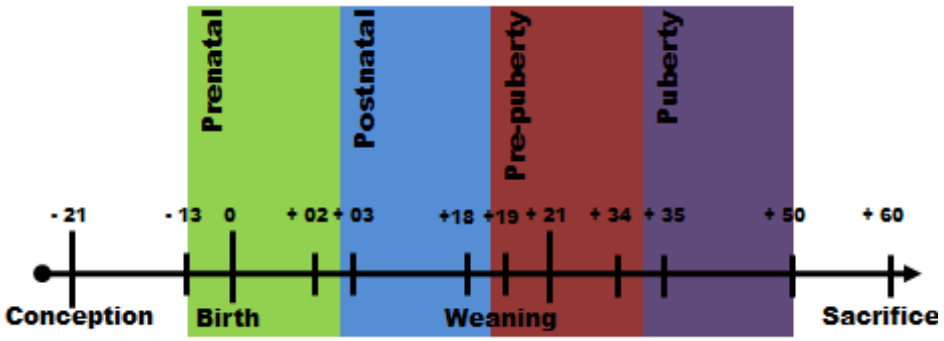

Figure 3-2: Schematic representation of the treatment groups according to age.

MA was dissolved in saline and administered s.c. to all rats within each of their 16 day age periods. With the exception of the prenatal group, all groups received two s.c. injections per day (4 h apart) for 16 days. The prenatal group received a 5 mg/kg injection once daily for 16 days. Administration of drugs for four treatment groups was given as follows:

1. Pre-natal (ND-13 to ND+02)
   Pregnant dams were injected subcutaneously behind the neck once daily with either vehicle or the doses MA specified in Table 3-1. On ND-13 until ND0 pups received drug via placental perfusion *in utero*, and on ND+01 to ND+02 pups received drug via lactation.
2. Post-natal (ND+03 to ND+18)
   Pups were injected subcutaneously behind the neck twice daily with either vehicle or the doses MA specified in Table 3-1.
3. Prepuberty (ND+19 to ND+34)
   Pups were injected subcutaneously behind the neck twice daily with either vehicle or the doses MA specified in Table 3-1.
4. Puberty (ND+35 to ND+50)
   Young rats were injected subcutaneously behind the neck twice daily with either vehicle or the doses MA specified in Table 3-1.

On ND+60 all rats were subjected to behavioural assessment (described below), after which the rats were euthanised via decapitation.

3.3.2 Neurotoxic dose regimen for pregnant dams and pups

Foetal mortalities reported in prenatal MA studies prompted a closer look at the toxicity threshold of pregnant dams and new-born pups. The best results on administering MA were found in studies that used an escalating dose regimen, promoting tolerance to the hyperthermic

and sympathomimetic effects of the drug, effectively lowering the mortality rate (Kuczenski *et al.*, 2007). The pregnant dams received a fixed daily dose which was chosen upon consideration of the results of previous studies (see Table 3-2) (Acuff-Smith *et al.*, 1996, Melo *et al.*, 2006, Šlamberová *et al.*, 2006, Williams *et al.*, 2004 ; Kuczenski *et al.*, 2007). The administration of MA to a pregnant dam is documented to produce a plasma level in the foetal brain similar to those found in human infants (Won *et al.*, 2001).

Table 3-2: Table of chronic MA administration of previous studies

Dosing regimen	**Reference**
20, 15, 10, 5, 0 mg/kg twice daily (8 h apart) On gestational days 7-12 or 13-18 (s.c.)	Acuff-Smith *et al.*, 1996
5 mg/kg daily On gestational days 8-22 (s.c.)	Melo *et al.*, 2006
5 mg/kg d for the duration of gestation (s.c.)	Šlamberová *et al.*, 2006
5, 2.5, 1.25, 0.625 mg/kg four times a day (2 h apart) On postnatal days 11-20	Williams *et al.*, 2004
Escalating dose regimen: 0.1-4 mg/kg throughout the treatment period, three administrations per day for 14 days followed by 6 mg/kg four times a day (4 h apart) for 11 successive days (s.c.)	Kuczenski *et al.*, 2007 (adult rats)

3.4 Behavioural tests

The tests used to measure cognitive function, locomotion and depressive-like behaviour are described in the following section and respectively include the NOR test, OFT and the FST. In order to avoid interference of any of the tests with the subsequent ones, a minimum of 30 min between testing times is required, similar to the acclimatisation period before each test. This has been validated by Blokland and colleagues (Blokland *et al.*, 2012).

3.4.1 Novel object recognition test

The NOR test is a well-documented, validated test of declarative memory in rodents, which relies on the premise that rats prefer to explore a novel object relative to a familiar one. The NOR test has relevance for depressive disorders as a measure of cognitive function that is known to be affected in depressed patients (Ennaceur & Delacour, 1988).

The NOR test is designed to measure working memory and cognitive function, and is based on an object recognition task. Recognition memory is divided into 3 phases: acquisition,

consolidation and retention and is influenced by D_1 and D_2 receptor types in the nigrostriatal pathway. This type of memory can be measured in the NOR test. D_1 receptor agonists are documented to enhance recognition memory retention for 72 h post in the NOR test-treatment, as compared to a vehicle control group in the NOR test (de Lima *et al.*, 2011). MA is known to increase DA neurotransmission in the nigrostriatal pathway (among other regions) and can also cause degeneration of dopaminergic nerve terminals when neurotoxic doses are ingested (as discussed in Chapter 2). Whether the damage to DA terminals is long-term is yet to be shown. These background information prompted the implementation of the NOR test in this study.

For the current study the NOR test was carried out as described previously (Grayson *et al.*, 2007; McLean *et al.*, 2010). Two days prior to testing (ND+58), the rats were exposed to a 10 min habituation session in the NOR box of dimensions 500 mm x 500 mm and 400 mm high, made of opaque, solid black Perspex by the Instrument Making Department of the Faculty of Engineering, NWU. The experimental trials were conducted on the third day (ND+60), when each rat was exposed to two identical immovable objects (EA1 and EA2) inside the box (see Figure 3-3) for a period of 5 min (T1). The rat was then returned to its home cage for a 90 minute inter-trial interval. The box and objects were then cleaned and wiped with 20% ethanol to remove any odour. One of the two objects was replaced with an object of a different size, shape and colour, unfamiliar to the rat (i.e. novel object). Each rat was allowed to explore both the novel and familiar objects inside the test box for a 5 min retention trial (T2). All experiments were video-documented by a camera mounted above the test box and the videos were analysed for differences in object exploration time. Object exploration by the rat is defined as sniffing, licking or touching the objects with forepaws (Grayson *et al.*, 2007). The exploration times of each object in each trial were recorded manually using a stopwatch. The time exploring the novel object (seconds) was calculated.

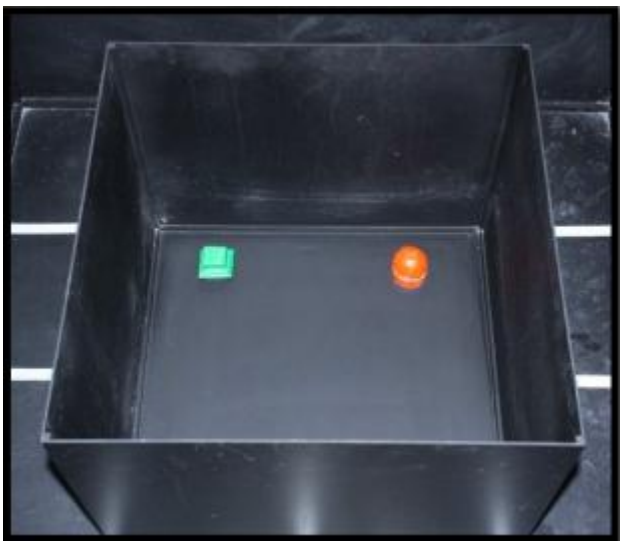

Figure 3-3: The novel object recognition test area in which two different test objects are presented. Dimensions: 500 mm x 500 mm x 400 mm with an open top and the two coloured objects used in the test.

The NOR test is not stressful for the rat because of its natural preference to approach and explore novel objects rather than familiar ones. The NOR test is non-invasive, low stress and easy to perform, requiring relatively little time. No additional training or other processes are needed. It measures short-term/working memory in rats and in particular their ability to distinguish between the familiar and the unfamiliar. The time spent exploring the novel object during T2 is an indication of short-term memory function. If the rat spends more time exploring the novel object, it implies that it remembers the previous object from the first exposure, but if the rat spends an equal amount of time exploring both the objects, it is assumed that it has no recollection of the object from the first exposure (Mathiasen *et al.*, 2010).

3.4.2 Open field test

The OFT allows the measurement of locomotive behaviour and anxiety. The OFT has little predictive validity for anxiety disorders, as can be seen from the sensitivity to effective treatments of anxiety disorders, such as alprazolam and SSRIs. However, the sensitivity to classical anxiolytics (benzodiazepines) that are used as treatment for general anxiety makes the model valid for the measurement of stress-induced anxiety (Prut & Belzung, 2003). The FSL rats are a valid model for studying depressive-like behaviour and also exhibits anxiety-like behaviour when stressed beforehand. FSL behaviour in the OFT presents as less active behaviour (decreased line crossing), corresponding with psychomotor retardation in humans with MDD (depressive-like behaviour) (Neumann *et al.*, 2011). The FSL rats do not exhibit significant increases in anxiety when subjected to the elevated plus maze test, however when subjected to the social interaction test, a significant increase in social anxiety is observed. MDD

is highly co-morbid with anxiety-related disorders and therefore anxiety is sometimes measured in models of depressive-like behaviour (Neumann *et al.*, 2011). Although depressive symptoms of MDD is successfully treated with SSRIs (among other antidepressants) in humans and in animal models, any decreased anxiety-like behaviour in animal models is not detected in the OFT. Therefore the sensitivity of the OFT and the type of anxiety comes into question upon measuring the behaviour of the FSL rats and in the current study, the OFT will only be used to measure motor function and cognition.

The OFT is a standard, validated test to measure locomotor activity in rodents. This test is based on the observation that rats have a natural curiosity, driving exploration of open spaces, yet they find open spaces aversive due to a risk of vulnerability to potentially present predators. Protectively enclosed or sheltered spaces, for example closer to a wall, are perceived by the rat as safer. The OFT consists of a novel, enclosed space (to prevent the rat jumping out or moving away from the space) into which the rat is placed for a period of 5 min and its movements monitored (Dubovicky & Jezova, 2004; Lynn *et al.*, 2010). The dimensions of the enclosed space are a breadth and depth of 100 cm each and a length of 50 cm. The floor of the space is divided into sixteen squares, twelve outer ones and four central squares. This is done by six lines, each 25 cm away from the walls and each other. At the beginning of the test each rat is placed in the same position on the field (the center) and monitored from there (Lynn *et al.*, 2010).

3.4.3 Forced swim test

The FST was designed to measure depressive-like behaviour, being captured by immobility of the rat as a type of despair (Castagne *et al.*, 2010). The classical FST requires a preconditioning 15 min trial of swimming 24 h prior to the scoring 5 min trial, thereby to induce a state of despair. FSL rats display inherent depressive-like behaviour, i.e. immobility in the FST, relative to FRL rats even in the absence of a preconditioning trial. Immobility behaviour is a depressive-like behaviour observed in stress-sensitive rats and is documented to be an indication of psychomotor retardation observed in depressed patients (Neumann *et al.*, 2011; Overstreet *et al.*, 2005). The method has been adjusted by Cryan and colleagues to an increased water depth (30 cm) and behavioural scoring in 5 s intervals so as to be able to discriminate finely between behaviours. It is divided into three measurable, active behaviours (immobility, swimming and climbing) to increase the sensitivity of the test to be able to detect different behavioural effects of the monoaminergic neurotransmitters (Cryan *et al.*, 2002). SSRIs such as fluoxetine target 5HT reuptake selectively and is documented to increase the frequency of swimming behaviour with

no effect on climbing behaviour. In contrast, NE-reuptake inhibitors such as maprotiline that selectively increase NE levels increase climbing behaviour without affecting swimming behaviour. Both antidepressants however are associated with decreased immobility behaviour and therefore decreased depressive-like behaviour (Detke & Lucki, 1996). Optimal use of the FST requires a water depth of at least 30 cm that the rat will not be able to touch the bottom of the water-filled cylinder. Studies that use less than 30 cm water depth are not able to differentiate between serotonergic and noradrenergic behaviours and also records less climbing behaviour. The ability of the rat to touch the bottom of the cylinder discourages swimming and climbing behaviour and appears as immobility behaviour (Detke & Lucki, 1996). The FST can also distinguish drugs with no antidepressant effects, such as the benzodiazepines (with the exception of alprazolam). These drugs have no effect in the FST (Cryan *et al.*, 2005). False positive results can occur when psychomotor stimulants are still in the systemic circulation of the rat.

The FST was developed to measure depressive-like behaviour in rats and mice and is one of the widest used tests for antidepressant screening. The rats are placed in a transparent Perspex® cylinder from which they cannot escape (60 cm high and 25 cm in diameter). The cylinder is filled to 30 cm with water kept at a temperature of 25˚C ± 2˚C. The three activities/behaviours that can be measured are defined as:

- Swimming: movement across the surface, moving around in the cylinder.
- Climbing: upward movements, clawing against the side of the cylinder, attempting to get out.
- Immobility: assuming an immobile position/floating, doing only what's necessary to keep head above water and nothing else.

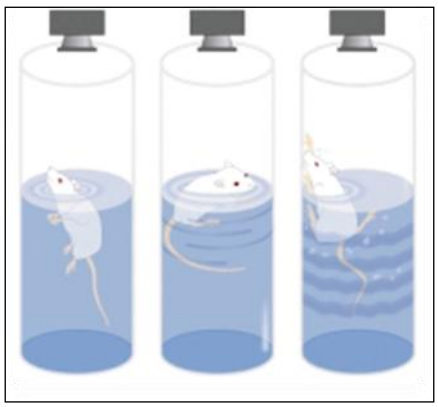

Figure 3-4: Immobility, swimming and climbing behaviour in the forced swim test (Cryan *et al.*, 2002)**.**

The light intensity was kept below 200 lux. The rats were recorded for 7 min of which only 5 min were scored (the first and last minutes were omitted). The scoring was done by recording the test and later evaluating and categorising the results. The immobility measured was classified as a sign of 'learned behavioural despair'. The rat adopted an immobile pose to conserve energy when it learned that escape is not possible, it moved only to keep its head above water. This was interpreted as giving up hope (despair) (Castagne *et al.*, 2010; Cryan *et al.*, 2002). Activities such as swimming and climbing could also be measured in an attempt to increase the sensitivity of the test. With an increase in serotonergic activity there was an increase in swimming activity. With an increase in noradrenergic activity an increase in climbing activity is observed (Cryan *et al.*, 2002).

3.5 Statistical analysis

The data were analysed using Student's t-test (unpaired, two-tailed) and Cohen's test for effect size. Graphpad Prism version 6 for windows (Graphpad software, San Diego, USA) was used for the statistical analysis and graphical presentations. The Statistical Consultation Service of the North-West University was consulted to verify the appropriateness of statistical analyses. Data are expressed as the mean ± S.E.M. and a value of $p<0.05$ was considered statistically significant, where * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. Data analysed using the Cohen test were expressed as $d>0.8$ denoting practical significance, where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). The number of animals used in each group (n) is indicated on their respective graphs.

4 Chapter 4 Results

This Chapter provides the data and analyses of the results from behavioural assessments performed on FSL and FRL rats at ND+60, following early-life treatment with saline (vehicle) or MA. The data were derived from the FST, NOR test and the OFT, as discussed in Chapter 3. The results are presented and key observations pointed out in this Chapter, with an in-depth discussion thereof presented in Chapter 5.

Importantly, the results from the current M.Sc. study were compromised by renovations at the animal centre at the North-West University, so that rats were moved to the National Health Laboratory Service (NHLS) in Edenvale, Gauteng, which also had issues with environmental dysregulation, consequently introducing the risk of an unpredictable long-term stressors into the study with resulting adverse effects in the rats. This undesirable yet unavoidable event resulted in poor breeding, leading to insufficient animal numbers in certain test groups, and hence poor statistical power of the data. The stress also negatively affected behaviour in the animals, so that the reliability of the data may have been compromised to some extent.

Data obtained from the FST, the NOR test and the OFT with corresponding behavioural parameters and aspects evaluated are presented in Table 4-1 of which the data sets will be presented in the following sections.

Table 4-1: Summary of the components of the behavioural tests discussed in this section.

Behavioural test	Parameter	Unit of measurement	Aspect evaluated
FST (forced swim test)	Immobility	Time spent immobile (seconds)	Depressed mood
	Swimming	Time spent swimming (seconds)	Serotonergic-related activity
	Climbing	Time spent climbing (seconds)	Noradrenergic-related activity
NOR (novel object recognition test)	Exploration	Time spent exploring the novel object (seconds)	Cognitive disturbance/impaired memory function
OFT (open field test)	Line crossing (locomotion)	Number of lines crossed	Locomotor activity (psychomotor retardation)

4.1 Results from the forced swim test

4.1.1 Validation of the FSL rat model

To demonstrate significant stress-sensitivity of the FSL rats (as discussed in Chapters 2 & 3) in the experimental setup of the current study, saline-treated FRL and FSL rats were tested in the FST, and their depressive-like behaviours compared. Figure 4-1 shows the immobility behaviour as displayed by FSL and FRL rats at postnatal day 60 (ND+60) following the indicated early-life treatment with saline (vehicle control).

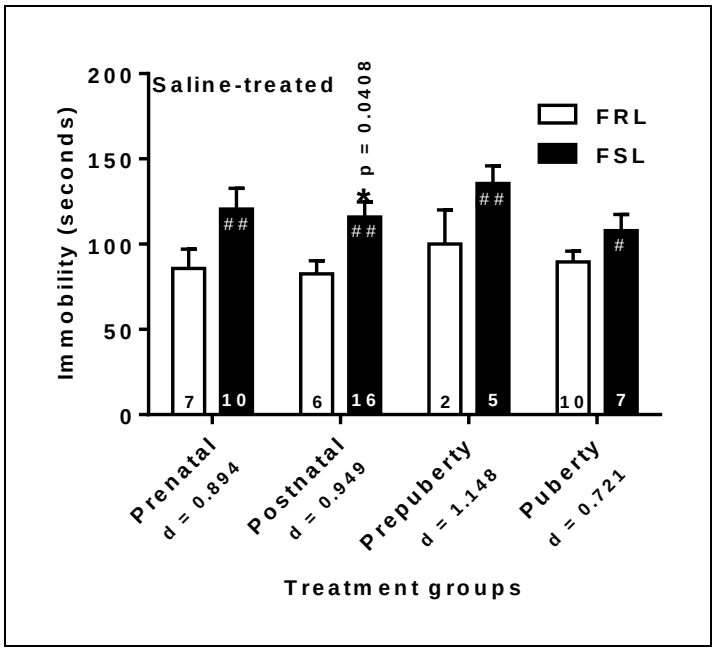

Figure 4-1: Immobility behaviour of FRL and FSL rats as measured in the FST performed on ND+60, following early-life administration with saline at the indicated age periods (prenatal, postnatal, prepuberty and puberty). Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant, where * = $p<0.05$. In addition, data was analysed using the Cohen test for effect size. In this case, $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). The number of animals used in each group (n) is indicated on the respective bars.

As can be seen in Figure 4-1, there were practically significant increases in immobility behaviour in FSL vs. FRL rats regardless of the age of exposure to injection stress (saline), which signifies a trend for enhanced depressive-like behaviour in FSL rats compared to FRL rats. However, this did reach statistical significance in the postnatal group.

As mentioned previously, the reliability (including statistical power) of the data has been compromised, probably due to the presence of unpredictable environmental stress and a reduced number of animals used in this study ($n<16$ per group). Nevertheless, the observed trend

towards increased immobility in FSL rats as compared to FRL rats was consistent across all age groups. To demonstrate a significant difference between FRL and FSL rats (i.e. that FSL rats display depressive-like behaviour relative to FRL rats), appropriate data sets can be combined to increase the number of data points per treatment group. Rats treated during the postnatal, prepuberty and puberty phases were all directly subjected to injection stress, whereas in the case of the rats treated during the prenatal phase only the pregnant dams were injected. Therefore, the data from rats treated during the postnatal, prepuberty and puberty phases were pooled and the data re-analysed. A presentation of the pooled data can be seen in Figure 4-2.

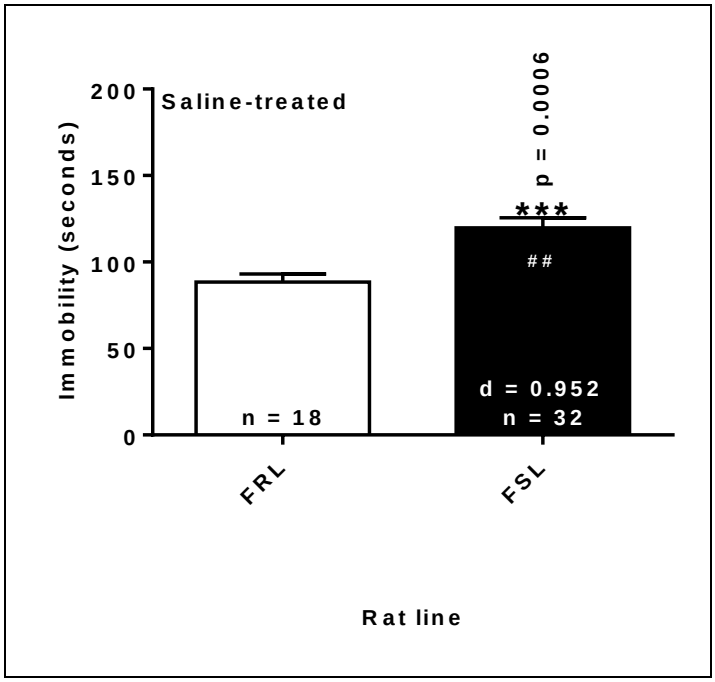

Figure 4-2: Pooled immobility behaviour data for FRL and FSL rats as measured in the FST performed on ND+60, using data from animals treated during the postnatal, prepuberty and puberty phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare the FSL and FRL rat groups. $P<0.05$ was considered statistically significant, where *** = $p<0.001$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance). The number of animals used in each group (n) is indicated on the graph.

These pooled data show a practically *and* statistically significant increase in the immobility behaviour of saline-treated FSL rats compared to FRL rats. This demonstrates inherent depressive-like behaviour in FSL rats, thus validating the FSL model under our experimental conditions.

4.1.2 MA-induced changes in immobility behaviour

Figure 4-3 depicts immobility behaviour observed in FSL vs. FRL rats on ND+60 before and after the indicated early-life chronic administration of MA or saline (vehicle control).

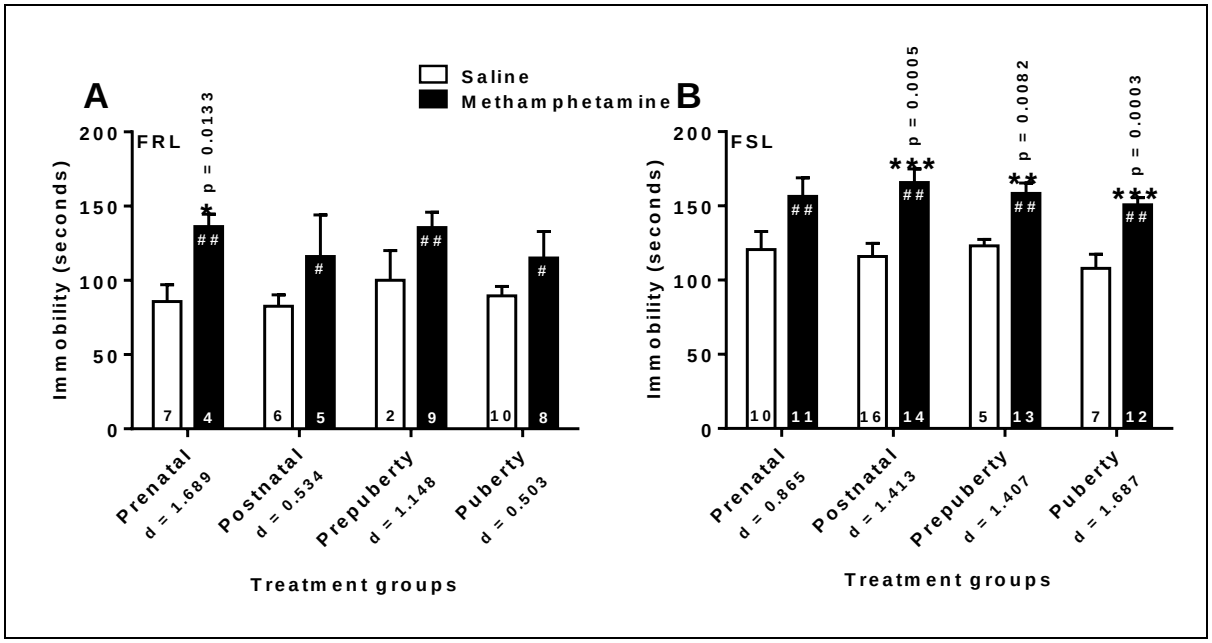

Figure 4-3: Immobility behaviour of saline and MA-treated (A) FRL and (B) FSL rats as measured in the FST performed on ND+60, following early-life administration with either MA or saline at the indicated age periods. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant, where * = $p<0.05$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). The number of animals used in each group (n) is indicated on the graph.

Practically significant increases in immobility behaviour in all age groups of MA-treated FRL and FSL rats in comparison to their controls is evident as seen in Figure 4-3. These practically significant differences between data sets denote a trend toward increased immobility behaviour in both stress-sensitive and control rats, whereas statistically significant increases in immobility behaviour are seen in prenatal FRL rats and postnatal, prepuberty and puberty FSL rats relevant to saline-treated rats.

Similar to what has been described for Figure 4-3 above, Figure 4-4 depicts the pooled data of rats treated with MA during the postnatal, prepuberty and puberty phases.

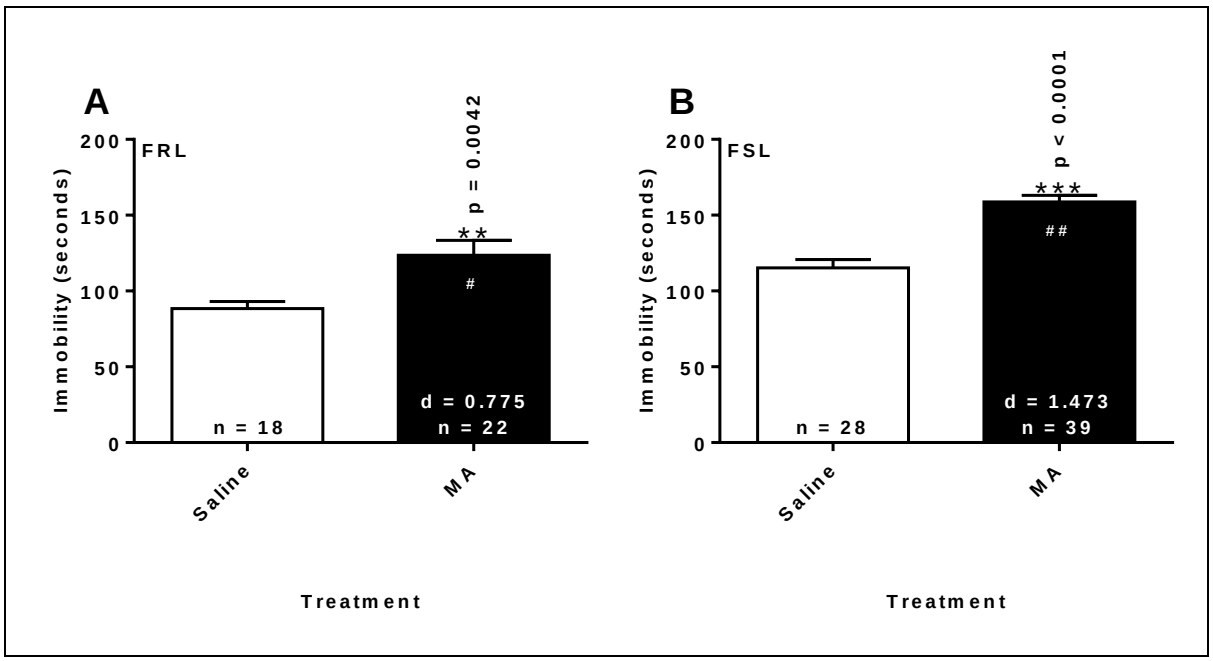

Figure 4-4: The pooled results of immobility behaviour of saline and MA-treated (A) FRL rats and (B) FSL, as measured in the FST performed on ND+60 following early-life administration with either MA or saline at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant, where ** = $p<0.01$; *** = $p<0.001$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). The number of animals used in each group (n) is indicated on the graph.

The pooled data as presented in Figure 4-4 reveals a practically *and* statistically significant increase in immobility behaviour in both MA-treated FRL and FSL rats as compared to their vehicle controls. Whereas FRL rats showed no statistically significant increases in immobility behaviour in the postnatal, prepuberty and puberty groups as seen in Figure 4-3, when these data were pooled and re-analysed, a practically and statistically significant increase in immobility behaviour for MA-treated rats is clear. Pooled (Figure 4-4) and separate data (Figure 4-3) confirmed a significant increase in immobility in FSL vs. FRL rats.

4.1.3 MA-induced changes in swimming behaviour

Figure 4-5 depicts the swimming behaviour of FRL and FSL rats in the FST on ND+60, following the indicated early-life exposure to saline (vehicle control) or MA.

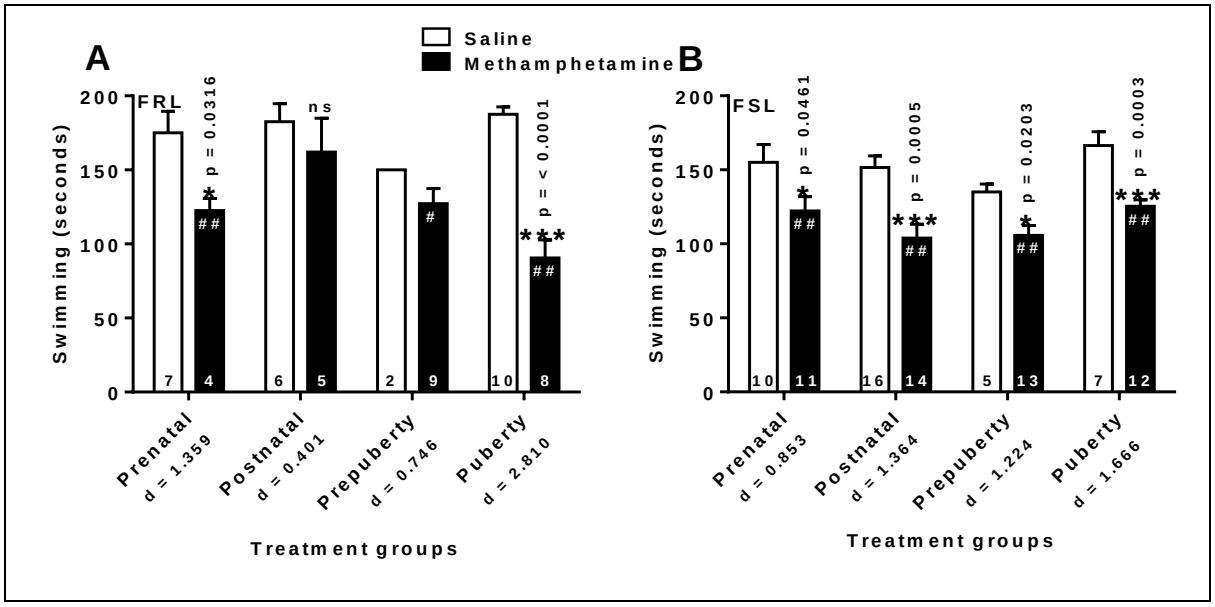

Figure 4-5: Swimming behaviour of all saline and MA-treated (A) FRL and (B) FSL rats as measured in the FST performed on ND+60; following early-life administration with either MA or saline at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant, where ** = $p<0.01$; *** = $p<0.001$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). No statistical or practical significance is indicated with: ns = not significant. The number of animals used in each group (n) is indicated on the graph.

In Figure 4-5B a practically and statistically significant decrease in swimming behaviour is seen in all MA-treated FSL rats, regardless of age of exposure. However, it can be seen in Figure 4-5A that the prenatal and puberty FRL groups exhibited practically and significantly decreased swimming behaviour and the prepuberty group exhibited practically significant decreased swimming behaviour. The practical significances of differences between data sets denote a trend toward decreased swimming behaviour in all MA-treated FRL and FSL rats. In general it can be seen that the decrease in swimming behaviour is more robust in FSL rats than in FRL rats.

Figure 4-6 depicts the pooled data of swimming behaviour of FRL and FSL rats treated during the postnatal, prepuberty and puberty phases.

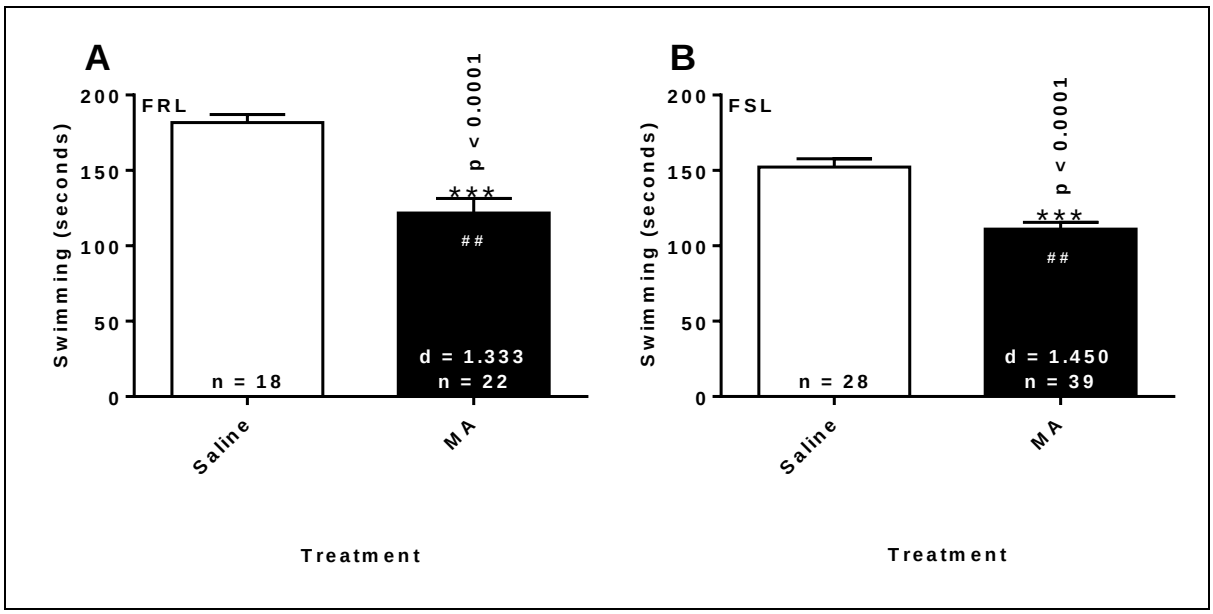

Figure 4-6: The pooled results of swimming behaviour of saline and MA-treated (A) FSL and (B) FRL rats as measured in the FST performed on ND+60; following early-life administration with saline or MA at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant, where *** = $p<0.001$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). The number of animals used in each group (n) is indicated on the graph.

The pooled results for swimming behaviour in Figure 4-6 describe a statistically *and* practically significant decrease in swimming behaviour for both FSL and FRL MA-treated rats when compared to their respective controls.

4.1.4 MA-induced changes in climbing behaviour

Figure 4-7 depicts the climbing behaviour of FRL and FSL rats in the FST on ND+60, following the indicated early-life exposure to saline (vehicle control) or MA.

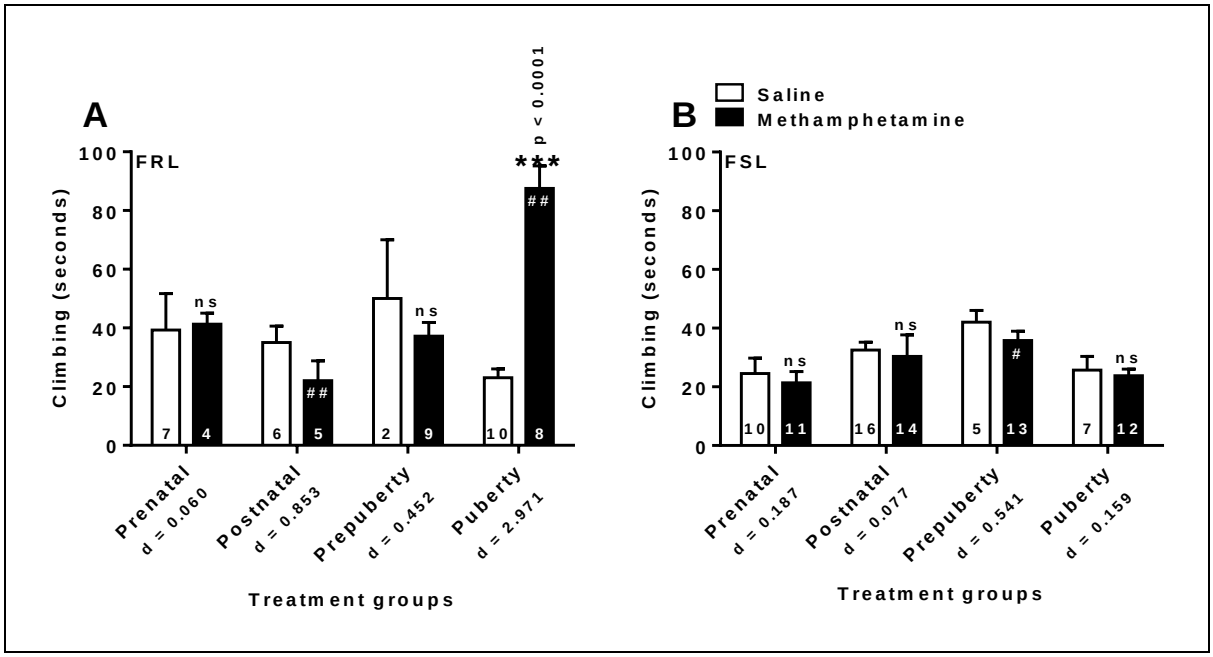

Figure 4-7: Climbing behaviour of saline and MA-treated (A) FRL and (B) FSL rats as measured in the FST performed on ND+60; following early-life administration of saline or MA at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant, where *** = $p<0.001$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). No statistical or practical significance is indicated with: ns = not significant. The number of animals used in each group (n) is indicated on the graph.

Statistically significant increases in the climbing behaviour of the FRL and FSL rats were found only in the puberty group of the FRL rats, as can be seen in Figure 4-7A. No statistically significant influence on climbing behaviour is evident in any of the FSL treatment groups. The practically significant differences between data sets are seen in the postnatal and puberty FRL groups and prepuberty FSL group and do not indicate any discernible trend.

Figure 4-8 depicts the pooled climbing behaviour of FRL and FSL rats in the FST on ND+60, following the indicated early-life exposure to saline or MA.

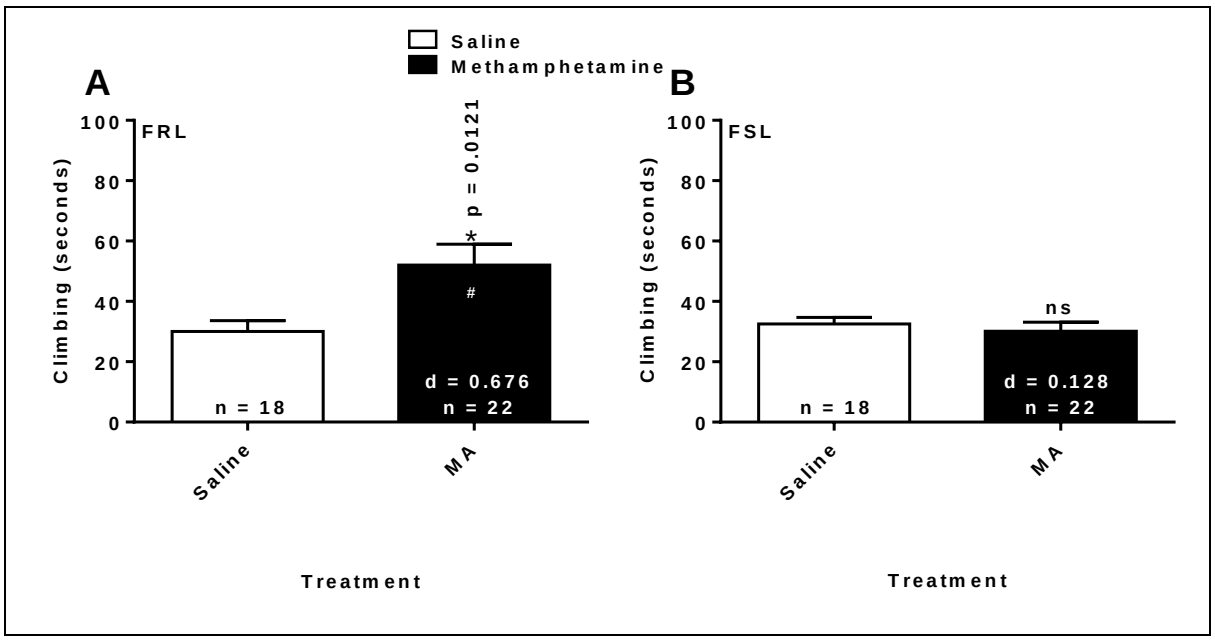

Figure 4-8: The pooled results of climbing behaviour of saline and MA-treated (A) FRL and (B) FSL rats as measured in the FST performed on ND+60; following early-life administration with saline and MA at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant, where * = $p<0.05$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where # = $0.8>d\geq0.5$ (medium practical significance). No statistical or practical significance is indicated with: ns = not significant. The number of animals used in each group (n) is indicated on the graph.

The pooled data of the climbing behaviour revealed a practically and statistically significant increase in climbing behaviour in MA-treated FRL rats but not in FSL rats. The effect in FRL rats may be explained by the distinctively large increase in climbing behaviour observed only in puberty-treated FRL rat group (compare Figure 4-7A), and it does not represent observations from rats in other FRL treatment groups.

4.2 MA-induced effects in the novel object recognition test

Figure 4-9 depicts the time spent exploring the novel object of FRL and FSL rats in the NOR test on ND+60, following the indicated early-life exposure to (vehicle control) or MA.

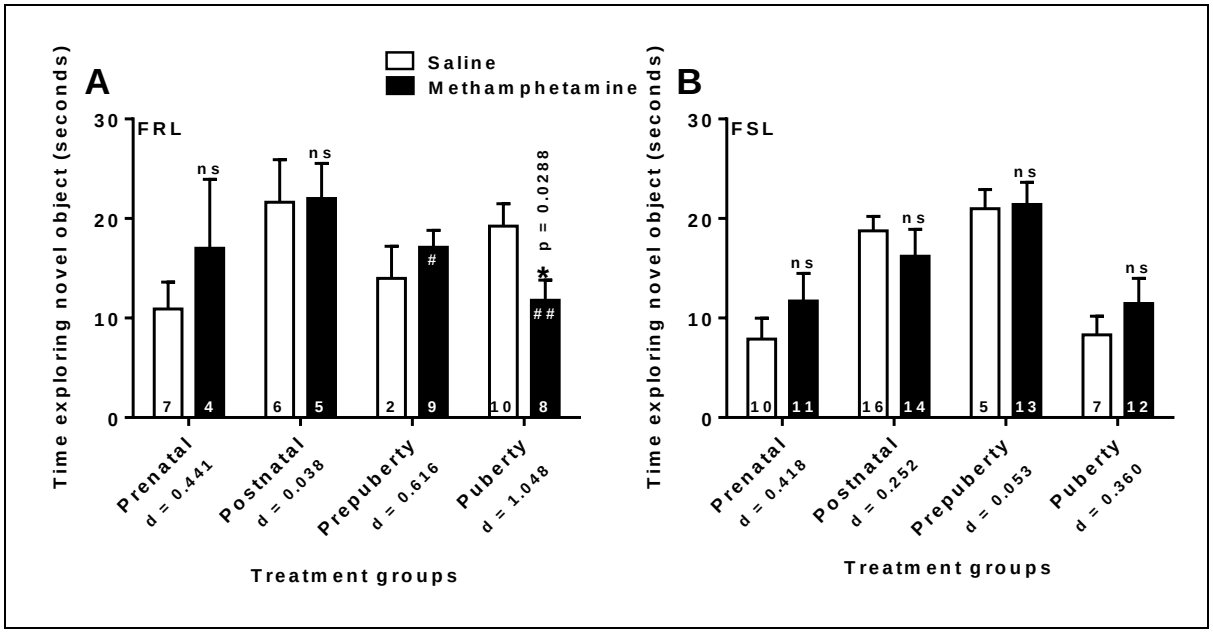

Figure 4-9: Time spent exploring the novel object in saline and MA-treated (A) FRL and (B) FSL rats as measured in the NOR test performed on ND+60, following early-life administration with saline or MA at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant where * = $p<0.05$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). No statistical or practical significance is indicated with: ns = not significant. The number of animals used in each group (n) is indicated on the graph.

No statistically significant differences in exploration time between vehicle control and MA-treated FSL or FRL rats are evident in any of the treatment groups, except for the puberty FRL group as seen in Figure 4-9A. The puberty FRL group exhibited a practically and statistically significant decrease in novel object exploration time, whereas the prepuberty FRL group showed a practically significant increase in exploration time. Of note is that no specific trend is detected among the age groups of either FRL or FSL rats.

Pooling of the NOR data of the postnatal, prepuberty and puberty treatment groups did not reveal any practically or statistically significant differences in exploration time, as can be seen in Figure 4-10. The statistical power of these data is insufficient to show whether the difference in cognition of saline- and MA-treated FRL and FSL rats is significant.

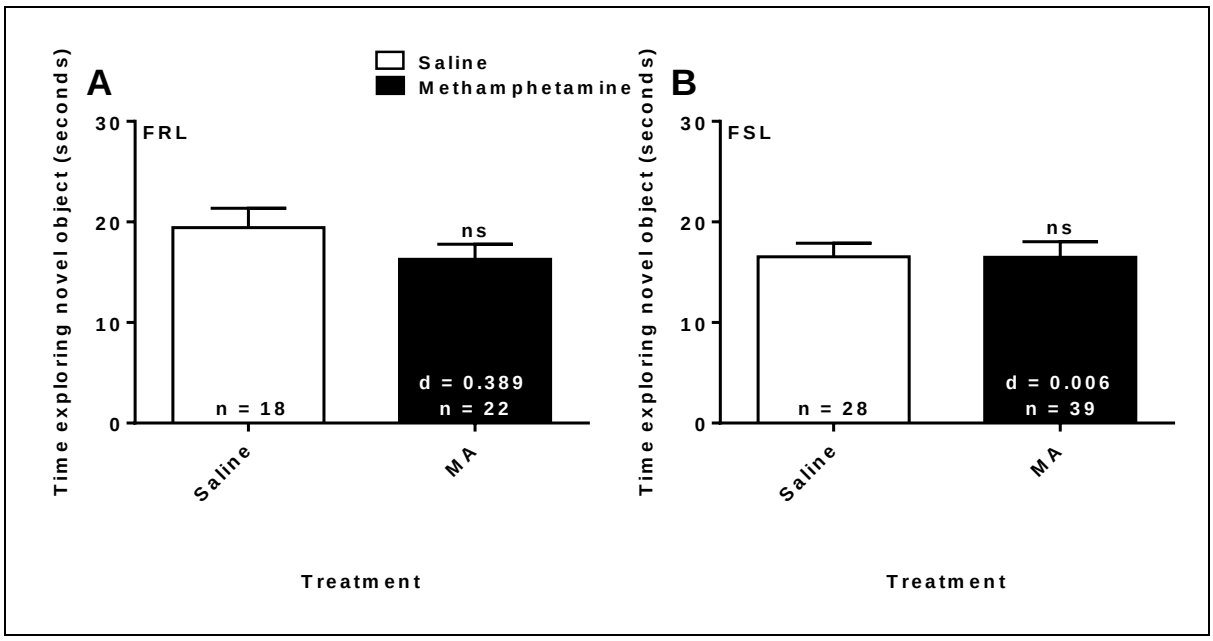

Figure 4-10: The pooled results of time spent exploring novel object of saline and MA-treated (A) FRL and (B) FSL rats as measured in the FST performed on ND+60; following early-life administration with saline and MA at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.5$ was considered practically significant. No statistical or practical significance is indicated with: ns = not significant. The number of animals used in each group (n) is indicated on the graph.

4.3 MA-induced changes in the open field test

Figure 4-11 depicts the lines crossed of FRL and FSL rats in the OFT on ND+60, following the indicated early-life exposure to saline (vehicle control) or MA.

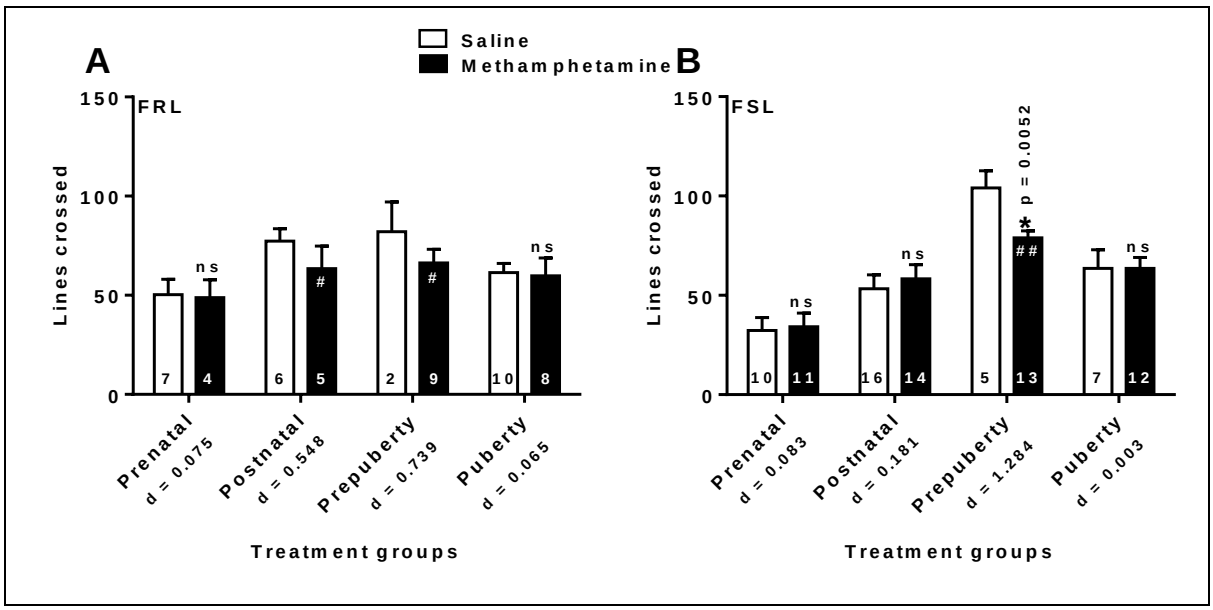

Figure 4-11: Lines crossed for saline and MA-treated (A) FRL and (B) FSL rats as measured in the OFT on ND+60, following early-life administration of saline and MA at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant, where * = $p<0.05$. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.8$ was considered practically significant where ## = $d\geq0.8$ (large practical significance) and # = $0.8>d\geq0.5$ (medium practical significance). No statistical or practical significance is indicated with: ns = not significant. The number of animals used in each group (n) is indicated on the graph.

Early-life exposure of MA did not statistically significantly alter the locomotor behaviour of any of the FRL treatment groups (Figure 4-11A) but practically significant decreases in locomotor behaviour is seen in postnatal and prepuberty FRL rats. Practically and statistically significant decreases in locomotor behaviour is seen in the prepuberty FSL rats as shown in Figure 4-11B. Pooling of the data of postnatal, prepuberty and puberty treatment groups did not reveal any practically or statistically significant differences (Figure 4-12). It should be recognised that the weakness of the data may have contributed to the current results.

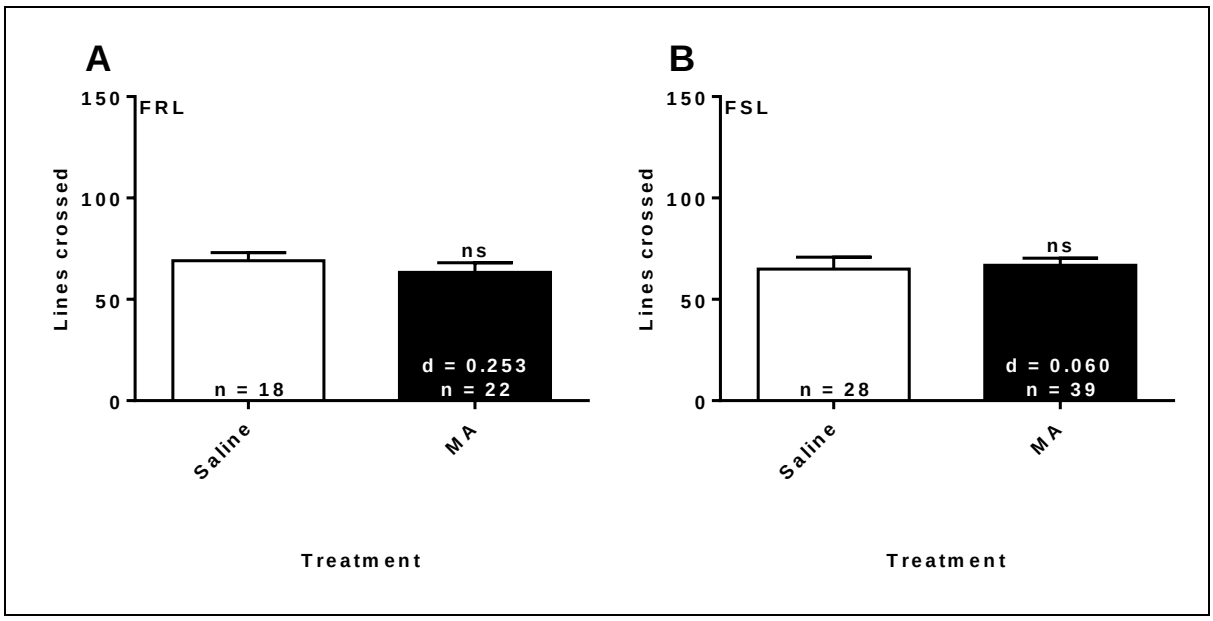

Figure 4-12: The pooled results of lines crossed of saline and MA-treated (A) FRL and (B) FSL rats as measured in the FST performed on ND+60; following early-life administration with saline and MA at the accumulative postnatal, prepuberty and puberty age phases. Data represent averages ± standard error of the mean (SEM). Data were analysed using the Student's t-test to compare each FSL rat group with its corresponding FRL rat group. $P<0.05$ was considered statistically significant. In addition, data was analysed using the Cohen test for effect size. In this case $d>0.5$ was considered practically significant. No statistical or practical significance is indicated with: ns = not significant. The number of animals used in each group (n) is indicated on the graph.

4.4 Summary of results

As a general observation the results from the current study are summarised in Table 4-2. The structure of the discussion in Chapter 5 will mirror that of the results presented in Chapter 4.

Table 4-2: Summary of significant effects of MA versus saline treatment in FRL and FSL rats

Behavioural Test	**FRL**	**FSL**
Forced Swim Test (FST)	**MA v. Saline**	**MA v. Saline**
Immobility	↑(prenatal)	↑(postnatal, prepuberty, puberty)
Swimming	↓(prenatal, puberty)	↓(prenatal, postnatal, prepuberty, puberty)
Climbing	↑(puberty)	↔
Novel Object Recognition test (NOR)	**MA v. Saline**	**MA v. Saline**
Time spent exploring novel object	↓(puberty)	↔
Open Field Test (OFT)	**MA v. Saline**	**MA v. Saline**
Lines crossed	↔	↓(prepuberty)

5 Chapter 5 Discussion, conclusion and recommendations

The discussion of results in this Chapter will identify and describe key observations from the data presented in Chapter 4, compare this with related literature findings and give a contextualised interpretation thereof.

As presented in Chapter 1, the aims of this study were to determine whether the behavioural outcome of MA exposure is dependent on the age of exposure, whether stress-sensitivity (congenital susceptibility) exacerbates the effects of MA exposure and whether early-life MA exposure can be linked to developing depressive-like behaviour, deficits in cognitive function and memory and deficits in locomotor behaviour later in life. This Chapter will also describe how these study aims have been addressed in the current project.

5.1 Discussion

The data discussed in this Chapter is undertaken with reference to statistically and practically significant differences between data sets. Besides calculating statistically significant differences between data sets, another useful approach to assist accurate interpretation of results of small data sets is to implement the Cohen-test (Cohen, 1988) to calculate effect size (d-value). An effect size of $d \geq 0.8$ is generally considered to denote a large practical significance, whereas $0.5 < d < 0.8$ is considered to indicate medium practical significance and $d \leq 0.5$ as indicating small/insignificant practical significance (Kirk, 2007). Bearing in mind that statistical significance does not suggest practical significance and vice versa, different meanings are construed from the data but the meanings complement each other to indicate the relevance and robustness of our findings. A short clarification for the use of these measures is presented.

Statistical significance is the focus of null hypothesis significance testing, and is concerned with whether results can be attributed to chance or sampling variability. It does not, however, signify the importance or magnitude of the difference between data sets, only that a difference can be found (Kirk, 2007; Thirthalli & Rajkumar, 2009). Any data set can have a significant p-value if the sampling size is large enough, but it would not mean that the difference is important or useful in a practical (or in this case, therapeutic) way. Practical significance (effect size), on the other hand, indicates whether the difference is large enough to be of value in a practical sense. Disregarding a sample because of lack of statistical significance would be very unwise if a large practical significance is present (Rosenthal *et al.*, 2000). Effect size is important in order to understand the magnitude or strength of the experimental findings (Kirk, 2007).

Statistical significance depends on 3 factors: magnitude of difference of the means, standard deviations of samples and number of subjects studied (Kalinowski & Fidler, 2010). In the current study, treatment numbers were low, which may account for the lack of statistical significance and can be misinterpreted as having little or no significance. Therefore, calculating effect size is paramount to the correct interpretation of the data. By pooling the data, the sample size is automatically increased, increasing the probability of finding a statistical significance in the difference between data sets (Thirthalli & Rajkumar, 2009). Even without statistically significant differences, practical significances may still be present in the pooled data, denoting value/magnitude and supporting the value/magnitude of the non-pooled data. Reporting of effect size is encouraged in publishing and required by some accredited journals (Kirk, 2007). Using this test along with the statistical significances of differences between data sets motivates the differences to be of significant value, especially in small data sets which would be less probable to reveal statistical significances. Therefore, the pooled data serves only to aid the interpretation of the non-pooled data sets, and not to be interpreted on its own.

5.1.1 Immobility behaviour as recorded in the forced swim test

Practically significant differences in immobility behaviour of all saline-treated FSL rats denoted a consistent trend to present with enhanced immobility behaviour compared to corresponding FRL control rats (Figure 4-1), as measured in the FST. This did not reach statistical significance in all instances. This was unexpected since FSL rats are known to display significantly enhanced depressive-like behaviour compared to FRL rats (Overstreet *et al.*, 2005). However, it was also evident that data numbers per treatment group were low and this could explain the lack of statistical significance of differences between data sets. When data were pooled (Figure 4-2), the difference between data sets was statistically *and* practically significant, as was initially expected in the first data set. This finding is also supported by similar data from previous studies in our laboratory using FSL rats (Brand, 2011; Liebenberg *et al.*, 2010; Mokoena *et al.*, 2010; Steyn, 2011) and in other laboratories (Abildgaard *et al.*, 2011; Neumann *et al.*, 2011 Overstreet *et al.*, 2005). Therefore, by pooling the data, the practically and statistically significant difference in immobility behaviour between saline-treated FSL and FRL rats is evident and validates the use of this translational model under our experimental conditions.

The next step was to evaluate MA-induced behavioural effects in FSL versus FRL rats. The data showed statistically and practically significant increases in immobility behaviour in the postnatal, prepuberty and puberty MA-treated FSL rats in comparison to their vehicle controls (Figure 4-3B), whereas practically and statistically significant differences were evident only in

the prenatal MA-treated FRL rats (Figure 4-3A). The development of depressive-like behaviour is well documented to manifest particularly during withdrawal after chronic MA abuse, when symptoms have been reported to be longer lasting (Chung *et al.*, 2010; McGregor *et al.*, 2005), although recovery is usually imminent (compare Chapter 2, section 2.4.4.1). The persistence of some MA withdrawal symptoms, however, may be a result from neurotoxic effects or underlying chronic changes (Davidson *et al.*, 2001). Numerous studies have documented changes of a structural (Colby, *et al.*, 2012 Chang *et al.*, 2007; Davidson *et al.*, 2001; Thompson *et al.*, 2004), metabolic (Chang *et al.*, 2007; London *et al.*, 2004; Thompson *et al.*, 2004) or behavioural (Davidson *et al.*, 2005; Williams *et al.*, 2002; Williams *et al.*, 2004) nature in animals and humans chronically exposed to MA. For example, the development of depressive-like behaviour as a result of psychostimulant withdrawal (Barr & Markou, 2005; Davidson *et al.*, 2001; McGregor *et al.*, 2005) and particularly during extended withdrawal (Ahmadlou *et al.*, 2013; Davidson *et al.*, 2001; Meredith *et al.*, 2005; Zweben *et al.*, 2004) is well documented in a variety of studies. Withdrawal is used in this discussion to describe the changes elicited by early-life MA administration on late-life behaviour, given that some symptoms of withdrawal may be due to neurobiological changes that appear to be enduring into later developmental phases (Grace *et al.*, 2012; Davidson *et al.*, 2001).

Furthermore, the effects of stress coupled with MA abuse are documented to cause enhanced neurotoxic effects (Raudensky & Yamamoto, 2007; Tata & Yamamoto, 2008). Therefore, MA-treated FSL rats were expected to exhibit significantly increased immobility behaviour in comparison with their saline-treated controls as a result of their genetic predisposition to stress-sensitivity. Whether the development of depressive-like behaviour is dependent on a predisposition to stress-sensitivity is not yet clear, however some studies document a higher prevalence of psychiatric symptoms in a MA-dependent population in comparison with the general population, regardless of previously experienced symptoms (McKetin *et al.*, 2006; McKetin *et al.*, 2011; Zweben *et al.*, 2004). The topic of stress-sensitivity is discussed in section 5.1.2 of the current Chapter. As before, the n-values per treatment group were low in the current study and could explain the lack of statistical significance between data sets. When the data were pooled (Figure 4-4), however, the MA-treated FRL rats also exhibited a practically and statistically significant increase in immobility behaviour compared to their vehicle controls, supporting the notion that MA exposure may be able to induce behavioural changes in both stress-sensitive and control rats.

The MA-induced increase in immobility did not reach statistical significance in the prenatally treated FSL rats (Figure 4-3B). This may be explained by the fact that pregnant dams received a milder MA treatment regimen than other age groups (5 mg/kg daily, compared to other groups receiving an escalating dose from 0.2 to 0.6 mg/kg twice daily – see Table 3-1), which may explain a less robust impact of MA exposure on depressive-like behaviour later in life in FSL rats treated in the prenatal phase. Nevertheless, age-specific differences in MA-induced neurodevelopmental effects have also been documented (Acuff-Smith *et al.*, 1996; Good & Radcliffe, 2011; Kokoshka *et al.*, 2000).

Prenatal MA exposure is documented to affect the development of specific brain structures and certain behaviours that persist into prepuberty (Chang *et al.*, 2004; Haefele, 2011; Šlamberová *et al.*, 2006). Little data are available regarding the effect of prenatal MA exposure on the development of depressive-like behaviour later in life. Focus is placed instead on the neurochemical changes elicited which often correlate with certain observed behaviours in MA-treated rats. MA toxicity to prenatally exposed groups is demonstrated by numerous studies reporting both pregnant dam and birth mortalities as well as decreases in gestational weight gain (Acuff-Smith *et al*., 1996; Kokoshka *et al.*, 2000; Kuczenski *et al.*, 2007; Vorhees *et al.*, 2000). Furthermore, MA exposure during critical postnatal developmental periods can alter the course of neurodevelopment and affect behaviour later in life. Clinical studies on this question, however, are fewer in number, but also provide clear evidence of MA toxicity and its association with the development of neuropsychiatric illness (Chung *et al.*, 2010; Plüddeman *et al.*, 2010; London *et al.*, 2004; Madide *et al.*, 2012; Smith *et al.*, 2008; Zweben *et al.*, 2004). An explorative study of children exposed to MA *in utero* conducted in the Cape Flats (Western Cape Province) in South Africa also revealed growth delays and notable differences in physical characteristics. These children were also described as having cognitive abnormalities and displaying deviant behaviour (Haefele, 2011). The association of MA exposure with mental health issues and particularly depression is supported by previous studies (Davidson *et al.*, 2001; Herbeck & Brecht, 2013; Plüddeman *et al.*, 2010; Rawson *et al.*, 2005).

In line with these reports, it was expected that the current study would reflect MA-induced development of depressive-like behaviours in rodents following MA exposure in all early-life treatment phases. In addition, it was expected that this response will be affected by congenital susceptibility so that stress-sensitive animals will yield a larger response to MA exposure. However, the data in the current study is too weak to provide a final answer on this hypothesis. We can therefore conclude that enhanced MA-induced depressogenic effects are found in both

FRL and FSL rats, with data also suggesting that such effects may potentially be more robust in FSL rats. The latter warrants further investigation.

5.1.2 Swimming behaviour as recorded in the forced swim test

In comparison with their vehicle controls, FSL rats displayed a practically and statistically significant decrease in swimming behaviour following MA exposure during prenatal, postnatal, prepuberty and puberty phases. Such a MA-induced reduction in swimming behaviour was not consistent in FRL treatment groups, with only the prenatal and puberty phase treatment groups showing a practically and statistically significant reduction in swimming behaviour (Figure 4-5). Decreased swimming behaviour has been linked to a decrease in serotonergic activity, suggesting that MA exposure inhibits serotonergic neurotransmission (Cryan *et al.*, 2002; Cryan *et al.*, 2005; Overstreet *et al.*, 2005). Indeed, MA has been found to profoundly modify serotonergic function (see below). The results on swimming behaviour with FSL rats were therefore expected. Since we noted a less robust effect on immobility in FRL rats compared to FSL rats (Figure 4-3), it was also not surprising to see a less robust effect on swimming behaviour in FRL rats compared to FSL rats. When the data were pooled (Figure 4-6), both practically and statistically significant decreases in swimming behaviour is seen in MA-treated FSL and FRL rats in comparison with their vehicle controls, suggesting a significant chronic effect of MA exposure on serotonergic neurotransmission.

Substantial proof of serotonergic toxicity is documented in numerous behavioural and neurochemical studies of chronic or acute neurotoxic MA exposure. Previous studies recorded serotonergic toxicity following an acute binge-administration of MA (Barr *et al.*, 2006; Cappon *et al.*, 1997; Fleckenstein *et al.*, 1997; Frost & Cadet, 2000) or during MA withdrawal (Brennan *et al.*, 2010; McGregor *et al.*, 2005; Renoir *et al.*, 2012). Long-term serotonergic toxicity after chronic MA exposure is also documented (Chung *et al.*, 2010; Meredith *et al.*, 2005). MA abuse has clinical implications regarding serotonergic toxicity both during early-life exposure and MA abuse later in life. As described in Chapter 2 (section 2.3.2), the serotonergic system matures rapidly during early development and plays an important role in the regulation of further neurodevelopment (Murrin *et al.*, 2007). 5HT also plays various important roles in anxiety and mood disorders, temperature regulation and the modulation of stress responses (Doyle & Yamamoto, 2010; Molinoff, 2012; Murrin *et al.*, 2007), so it can be expected that MA-associated disruption of serotonergic function will be associated with various mood-related disturbances.

Given that the stress-sensitive FSL rats appear to be significantly more affected by chronic MA exposure in the current data, the key role of 5HT throughout neurodevelopment is emphasised as

well as its effect on the regulation of further neurodevelopment (Murrin *et al.*, 2007), all of which may be expected to be impaired in stress-sensitive animals. In addition, excessive stress is documented to cause neuroinflammation (Black, 2002). MA exposure may also cause inflammation via activation of microglia (discussed in section 2.3.4), which may explain a stress-like effect by MA exposure. Furthermore, MA effects related to modulation of serotonergic neurotransmission include hyperthermia that, in combination with oxidative stress, leads to neurodegeneration (discussed in section 2.3.4). Stress has also been described to induce a hyperthermic response and the release of neuropeptides, one of which is corticosterone-releasing factor (CRF). As in humans, CRF is responsible for elevating blood corticosterone levels in rats (Black, 2002; Raudensky & Yamamoto, 2007), that in turn is capable of modulating the immune system. The serotonin type-2 ($5HT_2$) receptor is documented to mediate the release of corticosterone and hyperthermic responses to stress (Doyle & Yamamoto, 2010). Additional supporting literature describes the inflammatory hypothesis of depression, based on the development of depressive behaviour following the administration of pro-inflammatory cytokines (Smith, 1991; Walker, 2013). SSRIs are documented to possess anti-inflammatory properties, contributing to the notion that 5HT release plays a role in mediating the inflammatory process (Walker, 2013). Chronic exposure of MA is documented to cause damage to serotonergic neurons and the resulting lack of 5HT release may play a role in the presenting depressive-like behaviour (Fleckenstein *et al.*, 1997). Stress sensitive rats therefore seem to be more prone to serotonergic damage post MA exposure and the development of depressive-like behaviour than stress-resistant control rats.

5.1.3 Climbing behaviour

As a general rule, climbing behaviour was not affected in either FSL or FRL rats in any of the treatment groups, with one notable exception being the puberty MA-treated FRL rats (Figure 4-7). Increases in climbing behaviour have been documented in response to treatment with antidepressants active on catecholaminergic neurotransmission and is indicative of increased noradrenergic activity (Cryan *et al.*, 2005; Cryan *et al.*, 2002). Acute MA administration is documented to release large amounts of NE (Rothman *et al.*, 2001) that would present as significantly increased climbing behaviour in the FST. However, the behavioural assessment was executed 10 days after the last MA administration to ensure that no residual effects were present during testing. The data therefore suggest a long-lasting effect of MA exposure during puberty, an effect which is potentially dependent on genetic susceptibility.

MA is documented to release NE with a far greater potency than either DA or 5HT, with less evidence of lasting damage to noradrenergic neurons than dopaminergic and serotonergic neurons (Barr *et al.*, 2006; Easton *et al.*, 2007; Panenka *et al.*, 2013; Rothman *et al.*, 2001). Decreased DA and NE activity is documented to correlate with decreased climbing behaviour (Cryan *et al.*, 2005); therefore chronic MA exposure was expected to cause significantly decreased climbing behaviour later in life (D'Aquila *et al.*, 2012). When the data were pooled, as presented in Figure 4-8, the MA-treated FRL rats exhibited a statistically and practically significant increase in climbing behaviour. The practical significance of the difference between FRL data sets is moderate, and bearing in mind that the MA-treated puberty FRL group exhibited a high degree of practically significant increases in climbing behaviour, it would seem that the pooled data results from the puberty-treated FRL rat group does not represent the overall effect in all treatment groups.

Noradrenergic development is still in progress during rodent puberty (ND±40), making this system more vulnerable to environmental influence (Andersen, 2003; Herleniuz & Lagerkrantz, 2004; Murrin *et al.*, 2007). Why increased climbing is seen in adolescent control (FRL) and not in stress-sensitive (FSL) rats, is not clear and may be related to differences in the general neuronal resilience or pace of neurodevelopment of the two rat lines as discussed in section 2.2.5.

Long-term effects of chronic MA exposure on NE neurotransmission may be less prominent in comparison to its marked long-term effect on DA and 5HT neurotransmission, given that less lasting damage to the noradrenergic system is documented despite an increased capacity for NE release (Barr *et al.*, 2006; Easton *et al.*, 2007; Panenka *et al.*, 2013; Rothman *et al.*, 2001). The noradrenergic system, among others, is activated by stress (specifically social stress during puberty) (Bingham *et al.*, 2011) and also by MA exposure (Nordahl *et al.*, 2003; Barr *et al.*, 2006), suggesting a stress-like effect. A deficiency in DA and 5HT content as a result of neuronal damage is evident after acute, neurotoxic MA administration but is slowly, albeit partially, rectified over a matter of weeks (Frost & Cadet, 2000; Renoir *et al.*, 2012). A deficiency in NE levels is expected, seeing as MA releases NE to a greater degree than 5HT or DA (Rothman *et al.*, 2001). It is hypothesised that, because of the damage to neurons, the DA and 5HT neurotransmitter levels never reach full capacity again and therefore function at a lower level than before (Brennan *et al.*, 2010; Meredith *et al.*, 2005). Possibly, with the minimal damage of NE neurons, the activation of the noradrenergic system by the stressful environment of the FST may cause increased NE levels, resulting in increased climbing behaviour. These

increased NE levels during puberty could explain the greater level of increased climbing behaviour in comparison with postnatal, prepuberty and puberty FRL groups. All behavioural tests were carried out at an adult age, and therefore we conclude that MA exposure during puberty has in some way affected the noradrenergic system to respond differently to stressful events than any other age group. The magnitude of the difference between MA-treated and saline-treated FRL rats suggest that the NE system is sensitised by MA exposure and amplifies the noradrenergic response during stressful events such as behavioural testing.

5.1.4 Memory and cognition

Recognition memory, as measured by the NOR test, is unaffected in both MA-treated FRL and FSL rats compared to their vehicle controls (Figure 4-9) with the exception of the MA-treated puberty FRL group. Here the MA-treated FRL puberty group exhibited a practically and statistically significant decrease in novel object exploration time in comparison with their saline controls. It is interesting to note that this is the same group that showed enhanced noradrenergic neurotransmission (Figure 4-7). Overall, however, the data were not as expected, in view of the fact that numerous studies have documented cognitive deficits and learning difficulty in both rats and children exposed to MA during early-life development (Acuff-Smith *et al.*, 1996; Bubenikova-Valesova *et al.*, 2009; Domier *et al.*, 2000; Frost & Cadet, 2000; O'Dell *et al.*, 2011; Renoir *et al.*, 2012; Šlamberová *et al.*, 2006; Šlamberová *et al.*, 2010; Vorhees *et al.*, 2000; Vorhees *et al.*, 2009; Williams *et al.*, 2002; Williams *et al.*, 2004). Some studies focused on adult rats (instead of early-life effects) and acute neurotoxic doses (instead of chronic exposure), however all of the studies found significant deficits in recognition memory and cognition (Chang *et al.*, 2004; Cheng *et al.*, 2007; O'Dell *et al.*, 2011; Vorhees *et al.*, 2000 Kuczenski *et al.*, 2007; Williams *et al.*, 2002). The expected results were to find a significant decrease in the time spent exploring the novel object in rats treated with MA in comparison with their vehicle controls. However, previous studies have failed to describe definitive memory/cognition deficits in FSL rats when subjected to basal conditions (i.e. non-stressed, normally housed and non-treated rats). Furthermore, memory loss and cognitive deficit is described as a symptom of MDD, but is not one of the characteristics that the FSL rat shares with MDD (Naismith *et al.*, 2012; Overstreet *et al.*, 2005). However, previous studies documenting the effects of MA exposure on memory and cognition have found that MA causes learning deficits and cognitive impairment (Nordahl *et al.*, 2003; Vorhees *et al.*, 2000). Lower doses of MA are documented to enhance memory and cognition, and therefore it may be affected differently by neurotoxic doses (Meneses *et al.*, 2011). Although the current study had no definitive results linking cognitive impairment to MA abuse, cognitive impairment is described

as a symptom of stress sensitivity and as a symptom of MDD and can be considered to support the hypothesis of stress-sensitivity exacerbating MA-induced neurotoxicity (Naismith *et al.*, 2012; Swaab *et al.*, 2005). Specifically, deficits in memory and cognition were expected in the FSL rats. However, when the data were pooled, it showed no statistical (or practical) significance in the differences between data sets for either FSL or FRL rats.

A decrease in memory and cognitive function is documented in rats performing cognitive tasks after neurotoxic MA administration (Cheng *et al.*, 2007; Vorhees *et al.*, 2000; Vorhees *et al.*, 2009). The current data showed a significant difference in exploration time in FRL rats but not in FSL rats exposed to MA during puberty. Whether an increase or decrease in cognitive function is dependent on rat strain or age of MA exposure is not clear. This may relate to the differences in the expression and function of nicotinic acetylcholine receptors (nAChR) in the FSL and the FRL rat, which mediate DA release. Indeed, FSL rats already have an inbred change in cholinergic signalling, while nAChRs play a role in learning and memory (Auta *et al.*, 2000; Overstreet *et al.*, 2005). MA is documented to also have an effect on cholinergic systems (Hiranita *et al.*, 2006) and may therefore influence these receptors. Indeed nAChRs may be qualitatively different in FSL and FRL with respect to neurophysiology and in particular where inherent memory impairment may be expected in the FSL rat relative to the FRL rat. The data is not sufficiently strong to investigate this working hypothesis.

5.1.5 Locomotor activity

No statistically significant differences in locomotor activity, as measured in the OFT are evident in either MA-treated FSL or FRL rats compared to their vehicle controls (Figure 4-10) with the exception of the MA-treated prepuberty FSL group. The MA-treated prepuberty FSL rats exhibited practically and statistically significant decreases in locomotor activity compared to their vehicle controls. When the data were pooled, it did not reveal any statistical or practical significance between data sets for FRL or FSL rats. Increases in locomotor activity directly following MA exposure have been documented (Bubenikova-Valesova 2009; Grace *et al.*, 2010), as well as decreases following prenatal exposure to MA (Šlamberová *et al.*, 2007). Various changes in locomotor activity are recorded as a result of chronic MA exposure (Bubenikova-Valesova *et al.*, 2009; Suzuki *et al.*, 2004), acute neurotoxic MA administration (Good & Radcliffe, 2011) and also during MA withdrawal (Barr & Markou, 2005). Evidence of serotonergic and dopaminergic involvement in regulating locomotor behaviour and responses has been previously documented (Canales & Iversen, 2000; Jacobs & Fornal, 1995; Jacobs & Fornal, 1997). Decreases in the activity of these neurotransmitters may lead to decreased

locomotor activity as seen in the OFT. Furthermore, the reduced line crossings seen in the prepuberty MA-treated FSL rats suggest inhibited explorative tendencies, typical of stress-sensitive subjects (Overstreet *et al.*, 2005; Yadid *et al.*, 2000).

Importantly, when relating this back to the immobility data presented in Figure 4-3, reduced locomotor behaviour following MA exposure during prepuberty may have contributed to the corresponding immobility data. This is, however, not true for the enhanced immobility behaviour seen following MA exposure during postnatal or puberty phases (see Figure 4-3) and the data therefore suggest that the overall immobility data reliably reflects altered psychomotor and not locomotor activity. The increased immobility behaviour can therefore be attributed to increased depressive-like behaviour and not decreased locomotor activity. The change in locomotor activity in the prepuberty group, however, may be attributable to the sensitivity of the NE system during this phase of development (discussed in section 5.1.3). Interestingly, the change is seen only in prepuberty FSL rats and not puberty FSL rat, which may indicate a developmental difference between these two phases.

5.2 Conclusions

5.2.1 Outcomes of early-life MA exposure on behaviour in later life

Final conclusions of the study are presented below, also indicating how the study objectives of the study, as outlined in Chapter 1, have been met. We postulated that early-life MA exposure to FSL and FRL rats would affect neurodevelopment with resulting manifestation of depressive-like behaviour and deficits in memory acquisition and locomotor activity in later-life. Such deficits were postulated to be differently affected according to treatment age and more pronounced in subjects with predispositions to stress-sensitivity (FSL rats).

Significant differences in immobility and swimming behaviour were seen in the majority of FSL treatment groups, suggesting that MA potentiated depressive-like behaviour. These changes can be explained by putative MA-induced serotonergic toxicity in stress-sensitive FSL rats. No significant differences in immobility behaviour were evident in the FRL treatment groups, except in prenatally-treated rats, suggesting that MA exposure does not induce the development of depressive-like behaviour when genetic predisposition to stress-sensitivity is not present. Swimming behaviour is decreased only in prenatal and puberty FRL rats, suggesting serotonergic toxicity to be more prominent in FSL rats where all age groups exhibited decreased swimming behaviour. Only by pooling the data of postnatal, prepuberty and puberty groups can it be seen that significant decreases in immobility and swimming behaviour are present in both

FRL and FSL rats. These data indicate an overall depressogenic effect, possibly related to serotonergic toxicity, following early-life MA administration in FSL and FRL rats. Increased response to putative MA-induced serotonergic toxicity in FSL rats suggests a role for genetic stress-sensitivity to enhance susceptibility to MA-induced behavioural deficit. The FSL rat model of stress-sensitivity is therefore useful as a model of MA-induced development of depressive-like behaviour.

Climbing behaviour was affected only in the puberty FRL group, where it was significantly increased. The possibility that MA exposure sensitises the noradrenergic system during a particular developmental phase in which FRL rats are more vulnerable, is just one of many possible explanations and requires in-depth study to confirm it.

No discernible trend toward the effect of early-life MA exposure on memory and cognition in FRL and FSL rats is evident. The FRL MA-treated puberty age group displayed significantly decreased novel object exploration in comparison with saline-treated controls (Figure 4-9A). Besides the changes seen in the puberty FRL group, we found no evidence that MA exposure results in deficits in working memory and cognitive function in FRL or FSL rats during any stage of early development. Previous studies, however, reported changes in these behavioural parameters (Chang *et al.*, 2004; Cheng *et al.*, 2007; O'Dell *et al.*, 2011; Vorhees *et al.*, 2000). It is unclear whether these differences can be explained by the experimental conditions (early-life treatment), rat line used or inherent weaknesses in data required for statistical analysis, e.g. n-values, housing conditions or additional circumstances discussed previously.

No discernible trend of a significant effect of early-life MA exposure on overall locomotor activity in FRL and FSL rats is evident from the current study, with the exception of the prepuberty FSL group (Figure 4-11). Prepuberty may represent a developmental stage specifically vulnerable to the influences of MA exposure on locomotor activity. The changes in locomotor activity do not reflect in the immobility behaviour displayed in the FST, suggesting a psychomotor effect of MA exposure observable in the FST.

Significant differences in behaviour as a result of MA exposure during prepuberty and puberty would appear to be more prevalent across all behavioural tests (and in both rat lines) than the other age groups in the current study. MA exposure during prepuberty and puberty significantly altered cognitive function, swimming and climbing behaviour in FRL rats and the immobility and swimming behaviour of the FSL rats. Although these behavioural differences are not found exclusively in either of the FSL and FRL rats, they are more prevalent when compared to the

behavioural effects seen in the other age groups. When taking the current statistical power of the data into consideration, it remains remarkable that these robust trends could be identified.

5.2.2 Clinical relevance of key findings from this study

Chronic MA exposure during critical early-life neurodevelopmental stages may result in increased risk for developing mood disorders later in life. Genetic predisposition seems to play a key role in the detrimental neurodevelopmental effects of MA. MA exposure during adolescence will most likely elicit the most significant late-life effects. It is postulated that the neurodevelopmental effects of MA exposure relates strongly to serotonergic toxicity, and that the long-lasting depressogenic effects may relate to modulation in serotonergic, and noradrenergic neurotransmission to a lesser extent, but that is dependent on age and possibly on genetic susceptibility.

Different treatment strategies for MA withdrawal and dependence syndromes have been investigated with limited success (Karila *et al.*, 2010). To date no pharmacological compound is registered for use along with cognitive therapy in MA dependence. MA withdrawal is treated symptomatically with conventional antidepressants and antipsychotics, showing marginal reductions in MA use/relapse (Maglione *et al.*, 2000; Rawson *et al.*, 2002). SSRIs showed no efficacy in treating MA withdrawal due to powerful craving symptoms (Zorick *et al.*, 2011) and might even be contra-indicated in treatment of MA dependence (Shoptaw *et al.*, 2006; Karila *et al.*, 2010). Drugs that show some clinical efficacy in treating MA withdrawal all have a role in elevating monoamine concentrations (Barr & Markou, 2005), although drugs that elevate DA and NE appear more successful than those that elevate 5HT (Karila *et al.*, 2010; Zorick *et al.*, 2011). The course and underlying neuropathology of MA-induced depression is important for the development of novel treatment strategies, though age of exposure presents a different challenge (as is evident in the current study). Although the serotonergic system matures during early development and SSRIs are used to treat juvenile depression successfully (Murrin *et al.*, 2007; Wagner, 2005), the use of SSRIs is documented to be ineffective in MA dependence. Whether SSRI treatment will show a similar response in adolescent withdrawal-induced depression is not yet investigated.

5.3 Study limitations and recommendations

Foremost is that the quality of the study data was compromised by experimental conditions, rendering data with weak statistical power. As mentioned in the beginning of Chapter 4, the experimental phase of the study was relocated to the National Health Laboratory Service (NHLS) in Edenvale, Gauteng. This required the transportation of the rats to the NHLS

facilities, which we believe could have proved to be a stressful experience for the rats. This was corroborated by the significantly reduced litter numbers following the transportation of the animals so that an acceptable n-value for all groups could not be reached in the allotted time period (projected n-value per group: n=16) The temperature regulation and sound hygiene of the holding rooms were also suboptimal due to renovations at this institute. Rodent behaviour, and even more so for stress-sensitive FSL rats, is particularly sensitive to environmental stress, including that induced by noise and temperature fluctuations, all of which may have contributed to the weakness of the data obtained. Although many contributing factors may have influenced the accuracy of the results, a working FST model of depression was still evident from the data.

Rat strain effects could not be satisfactorily determined in the current study as a consequence of the inherent weakness of the data. The apparent robustness of the FSL data compared to the FRL data could not be demonstrated, although a trend is evident that genetic susceptibility may play a role (see section 5.1.2). Study on a larger scale is needed to confirm that stress sensitivity plays a key role in MA-induced development of depressive-like behaviour as a function of age. Establishing a definite link between stress-sensitivity and exacerbated MA-induced behavioural deficits could shed new light on preventative measures of MA dependence as well as introduce new grounds on which to investigate development of depressive-like behaviour. Nevertheless, the current data is still relevant as a preliminary indication of the role of early-life MA exposure in the development of depressive-like behaviour later in life, conveying the persistence of damage incurred during neurodevelopment as well as the stage-specific vulnerability to exposure.

To refine the interpretation of the data, biochemical markers to compare alongside the behavioural data, such as brain and blood norepinephrine (NE), dopamine (DA), serotonin (5HT), 3,4-dihydroxyphenylacetic acid (DOPAC) brain-derived neurotrophic factor (BDNF) levels and markers for oxidative stress can be analysed in future studies. Biomarkers such as BDNF levels and markers of oxidative stress may also give clarity on the neurobiological effects exerted by early-life MA exposure. Importantly, histopathological studies may also shed light on how neurodevelopment was affected.

The dose regimen for pregnant dams (prenatal exposure) could in future studies be similar to that used in postnatal, prepuberty and puberty rats, i.e. an escalating dose regimen. Additionally, the escalating dose regimen allows the rat to build a tolerance to the hyperthermic and sympathomimetic effects of MA, enabling it to survive a more neurotoxic dose. A dose-

response study of MA would therefore be beneficial to establish the optimum dose capable of producing evidence of chronic neurotoxicity.

The most prominent results were produced from MA exposure during the prepuberty and puberty phases, and therefore it may be beneficial to focus on these age groups in future studies. A wide array of changes takes place in that phase of development and would be more susceptible to external chemical and environmental influence.

Stress precipitates depressive-like behaviour in FSL rats (Neumann *et al.*, 2011; Overstreet *et al.*, 2005; Yadid *et al.*, 2000), an animal model for depression. It would be important to understand the wider spectrum of stress-related effects of early-life MA exposure. Also, it may be that MA induces a state of enhanced susceptibility to environmental stress, so that MA exposed rats may be more vulnerable to late-life stressors. As examples, social defeat and artificially induced inflammation are vital stress-induction methods in an animal model of depression, which may enhance MA-induced deficits in exposed animals. Different types of stress may have different outcomes on an animal model of depression, for instance, antisocial behaviour is a symptom of depression and therefore may be more vulnerable to social stressors than another form of stress, e.g. footshocks (Bingham *et al.*, 2011; Romeo, 2010). Stress is well documented to cause inflammation (Black, 2002), that in turn will increase neurodegeneration (Block & Hong, 2005). This artificially induced inflammation may be an adequate stressor with which to investigate the effects of MA exposure. The introduction of such a post-MA exposure stressor will allow us to extrapolate potential clinical consequences of MA-induced neurotoxicity. The evaluation of stress-sensitive vs. stress resistant rats alongside the introduction of a stressor could prove useful in elucidating the underlying mechanisms leading to anxiety-like and depressive-like behaviour.

Cognitive deficit is documented as a symptom in both MDD and MA-induced withdrawal (Herbeck & Brecht 2013), and possible involvement of elements of cognitive function such as nAChRs (see section 5.1.4) warrants further investigation. Another signalling system involved in memory formation is that of the glutamate-nitric oxide-cyclic guanosine monophosphate system (Brand *et al.*, 2012; Piedrafita *et al.*, 2007). It is interesting to note that this cascade is selectively affected in FSL vs. FRL rats, but that this difference is only observed in the presence of chronic stress (Brand *et al.*, 2012; Wegener *et al.*, 2010). The involvement of glutamate and nitric oxide (NO) in the process of MA-induced inflammation and neurodegeneration (section 2.3.4) provides a basis for investigating the possible role of this signalling pathway in the development of cognitive deficit in MA addiction.

It may be beneficial to perform the assessment on either ND+90 or ND+120 to determine long-term damage to dopaminergic neurons caused by binge-dosing of MA, as well as to determine the regenerative capacity of neurons. This will aid us in our investigation as to why results from post-mortem MA abusers do not correlate with data from animal studies (see Kitamura *et al.*, 2010).

6 References

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