Effects of chronic methamphetamine exposure during early or late phase development in normal and social isolation reared rats

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Abstract

Methamphetamine (MA) abuse is a fast growing drug problem, and is the second most widely abused drug world-wide. MA abuse has been linked to the development of symptoms indistinguishable from schizophrenia, referred to as MA psychosis. MA abusing individuals, who most often comprise adolescents and young adults, are 11 times more likely than the general population to develop psychosis. Of further concern is that *in utero* exposure to MA is also a growing problem, with more women addicts choosing MA as their primary drug. This has significant implications for the neurodevelopment of the child, with subsequent behavioural deficits later in life. Epidemiological studies suggests that *in utero* or early life MA exposure places a vulnerable individual at greater risk for developing schizophrenia, although this has never been formerly studied either at clinical or pre-clinical level. Animal models of early life adversity, such as post-weaning social isolation rearing (SIR), can assist in understanding the underlying mechanisms in MA abuse and vulnerability to develop MA psychosis.

The aim of the current study was to investigate the long term effects of either prenatal (*in utero*) or early postnatal administration of MA on the development of schizophrenia-like behavioural and neurochemical abnormalities later in life.

In the *in utero* study, pregnant female Wistar rats received either saline (Sal) or MA 5 mg/kg/day for 16 days by subcutaneous (s.c.) injection , starting on prenatal day 13 (PreND-13) up to postnatal day 2 (PostND02). Male offspring were selected for the study. On PostND 21, the animals were weaned and reared under group or isolation reared conditions for 8 weeks. In the early postnatal study, adult male Wistar rats were divided into group reared and SIR conditions from PostND21. Either group received an escalating dose of MA twice a day (0.2 mg/kg – 6 mg/kg s.c.) or Sal for 16 days, from PostND35 to PostND50. Both *in utero* and early postnatal groups were then subjected to various behavioural tests on PostND78, including assessment of social interaction (SI) and prepulse inhibition (PPI) of acoustic startle. Following behavioural testing, rats were sacrificed and brains snap frozen for later analysis of cortico-striatal monoamine concentrations, superoxide dismutase activity and lipid peroxidation.

In the prenatally exposed group no differences in %PPI was observed, although group reared animals receiving MA and SIR animals receiving Sal or MA showed a decrease in social interactive behaviours, including approaching, time together and anogenital sniffing. SIR animals receiving Sal or MA also showed a decrease in rearing. Regarding self-directed behaviours, group reared animals receiving MA and SIR animals receiving Sal or MA showed an increase in self-grooming. Although some disturbances in regional brain monoamines were

observed in the frontal cortex and striatum across the groups, this did not reach significance. A significant increase in malondialdehyde was observed in the striatum in group reared animals receiving MA as well as SIR animals receiving Sal or MA, indicating cell damage, possibly of redox origin.

In the early postnatal study, %PPI was significantly reduced in group reared animals receiving MA as well as in SIR animals receiving Sal or MA. Group reared animals receiving MA and SIR animals receiving Sal or MA showed a decrease in social interactive behaviours, including rearing, approaching, time together and anogenital sniffing. Regarding self-directed behaviours and locomotor activity, self-grooming and squares crossed was significantly increased in group reared animals receiving MA and SIR animals receiving Sal or MA. A significant increase in DA was evident in the frontal cortex of SIR and grouped housed animals receiving MA. DA in the MA + SIR combination was elevated but not significantly so. None of the treatments affected striatal monoamine levels. In the group reared animals receiving MA as well as the SIR animals receiving Sal or MA, a significant decrease in SOD activity was observed in the frontal cortex, indicating the presence of oxidative stress in this brain region. None of the parameters indicated an additive effect in MA + SIR treated animals.

In conclusion, prenatal exposure to MA led to some evidence of late-life behavioural and neurochemical abnormalities akin to schizophrenia, confirming its penchant for psychotogenic effects. However, chronic postnatal MA exposure was more emphatic, being as effective as SIR, a neurodevelopmental model of schizophrenia, in inducing deficits in the above-mentioned behavioural and neurochemical parameters. Thus, early adolescent abuse of MA is a significant risk factor for the later development of schizophrenia or psychosis. However, the risk appeared not to be exacerbated in a population at risk, i.e. in SIR animals.

Keywords: Social isolation rearing, methamphetamine, Wistar rat, prepulse inhibition, social interaction, psychosis, schizophrenia, monoamines, oxidative stress.

Opsomming

Metamfetamien misbruik (MA) is ‘n vinnig groeiende probleem en word beskou as die tweede mees gewildste dwelm wêreldwyd. Die misbruik van die dwelm word geassosieer met die ontstaan van psigiatriese simptome wat meestal nie van skisofrenie onderskei kan word nie, genaamd MA psigose. Individue wat MA misbruik, meestal tieners en jong volwassenes, is 11 keer meer geneig om psigose te ontwikkel as die normale populasie. Verdere kommer word gewek deur die feit dat baie menslike fetusse alreeds *in utero* blootgestel word aan MA, met meer dwelm misbruikende vrouens wat MA as primêre dwelm kies. Dit kan ‘n groot effek op die neuro-ontwikkeling van die kind hê, asook aanleiding gee tot gedrags afwykings later in die lewe. Epidemiologiese studies dui daarop dat *in utero* of vroeë blootstelling aan MA die risiko kan vergroot dat ‘n kwesbare individu skisofrenie sal ontwikkel, alhoewel dit nog nooit bestudeer is op kliniese of pre-kliniese vlak nie. Diere modelle van vroeë lewens ontbering, soos blootstelling aan sosiale isolasie (SI) na spening kan van hulp wees om die onderliggende meganismes van MA misbruik en kwesbaarheid om MA psigose te ontwikkel, beter te verstaan.

Die doelwit van die huidige studie was om langtermyn effekte van prenatale (*in utero*) blootstelling asook blootstelling aan MA tydens puberteit te bepaal op die ontwikkeling van gedrags en neurochemiese afwykings soortgelyk aan skisofrenie, later in die lewe.

In die *in utero* studie het swanger wyfies van Wistar rotte ‘n daaglikse subkutaneuse inspuiting van ‘n soutoplossing (Sout) of 5 mg/kg MA ontvang vir 16 dae vanaf prenatale-dag-13 (PreND-13) tot postnatale-dag 2 (PostND02). Uit die nageslag gebore is mannetjies uitgekies vir die studie. Diere is gespeen op PostND 21 en rotte is opgedeel in groepe blootgestel aan 8 weke SI of groeps-behuising. In die postnatale studie is volwasse manlike Wistar rotte opgedeel in groepe blootgestel aan 8 weke SI of groeps-behuising vanaf PostND 21. Groepe het dan of ‘n stygende dosis van 0.2 mg/kg tot 6 mg/kg subkutaneuse MA twee keer ‘n dag ontvang of Sout, vir 16 dae vanaf PostND35 tot PostND50. Gedragstoetse het vir beide die *in utero* en postnatale studie begin op PostND78 en het bestaan uit verskeie sosiale interaksie toetse en die persentasie prepulsinhibisie (%PPI) van die skrikreaksie. Na gedragstoetse is rotte onthoof en breine verwyder vir neurochemiese analise. Die vlakke van monoamiene in die frontale korteks en die striatum, die aktiwiteit van superoksied dismutase (SOD) en die vlakke van lipiedperoksiedase is bepaal.

In die prenatale groep was daar geen betekenisvolle verskille in %PPI nie, alhoewel groeps-gehuisveste rotte wat MA ontvang het, sowel as die SI rotte wat Sout en MA ontvang het ‘n betekenisvolle verlaging getoon het in sosiale interaksie, insluitend nader kom, tyd saam spandeer en tyd spandeer aan anogenitale snuif. SI diere wat Sout of MA ontvang het, het ook

‘n verlaging getoon in tyd op die agterpote staan. Ten opsigte van selfversorgings-bewegings het groeps-gehuisveste rotte wat MA ontvang het, sowel as die SI rotte wat Sout en MA ontvang ‘n betekenisvolle verhoging getoon in self-versorging, in vergelyking met groeps-gehuisveste kontrole diere wat Sout ontvang het. Alhoewel daar tekens was van veranderinge in monoamiene in die striatum en frontale korteks, was dit nie statisties betekenisvol nie. In die striatum was malondialdehied betekenisvol verhoog in groeps-gehuisveste rotte wat MA ontvang het, sowel as die SI rotte wat Sout of MA ontvang het, wat selskade aandui moontlik van redoks afwykings afkomstig.

In die postnatale studie is %PPI betekenisvol verlaag in die groeps-gehuisveste rotte wat MA ontvang het, sowel as die SI rotte wat Sout en MA ontvang het. Groeps- gehuisveste rotte wat MA ontvang het, sowel as die SI rotte wat Sout of MA ontvang het, het ‘n betekenisvolle verlaging getoon in sosiale interaksies, insluitend tyd op die agterpote staan, nader kom, tyd saam spandeer en tyd spandeer aan anogenitale snuif. In verband met selfversorgings-bewegings en lokomotoriese aktiwiteit is self-versorging en vierkante oorgesteek verhoog in groeps-gehuisveste rotte wat MA ontvang het, sowel as die SI rotte wat Sout of MA ontvang, in vergelyking met die kontrole groep. ‘n Betekenisvolle verhoging in dopamien (DA) was teenwoordig in die frontale korteks van groeps-gehuisveste rotte wat MA ontvang asook SI rotte wat Sout ontvang. In SI rotte wat MA ontvang was DA verhoog, maar nie statisties betekenisvol nie. Geen van die behandelings het monoamiene in die striatum geaffekteer nie. Groeps-gehuisveste rotte wat MA ontvang het, sowel as die SI rotte wat Sout en MA ontvang het, het ‘n betekenisvolle verlaging in SOD aktiwiteit in the frontale korteks getoon. Dit impliseer die teenwoordigheid van oksidatiewe stres in die brein deel. Daar was geen aanduiding van ‘n vergrote effek in MA+SI diere nie.

Ten slotte, prenatale blootstelling aan MA induseer sommige van die gedrags en neurologiese afwykings soos gesien in skisofrenie, wat MA se geneigdheid om psigose te induseer bevestig. Chroniese postnatale MA toediening was so effektief soos SI, ‘n neuro-ontwikkelings model van skisofrenie, om gedrags en neurologiese afwykings te induseer. Ons kan dus sê dat misbruik van MA gedurende die tienerjare ‘n groot risiko inhou om later psigose of skisofrenie te ontwikkel, alhoewel die risiko nie vererger is in ‘n kwesbare populasie bv. SI diere nie.

Sleutelwoorde: Sosiale isolasie, metamfetamien, Wistar rot, prepulsinhibisie, sosiale interaksie, psigose, skisofrenie, monoamiene, oksidatiewe stres.

“Do not go where the path may lead, go instead where there is no path and leave a trail”

Ralph Waldo Emerson

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"The fact is, that to do anything in the world worth doing, we must not stand back shivering and thinking of the cold and danger, but jump in and scramble through as well as we can."

Robert Cushing

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Chapter 1 - Introduction

This introductory chapter serves as an orientation to the dissertation and study as a whole, describing (1) the format of the dissertation (i.e. approach and layout), (2) the problem statement (concise literature overview, which is elaborated on in Chapter 2), (3) study objectives and (4) the study layout (experimental design/approach), and (5) hypothesis (expected results). All abbreviations are listed in Addendum F.

1.1 Dissertation approach and layout

This dissertation is presented in an article format, whereby the key data is prepared as manuscripts (see Chapter 3 and 4) for publication in selected scientific journals. All complimentary data not included in the article, but necessary for the study as a whole, is presented in an addendum (see Addendum B). A literature review, a general discussion and study conclusions are presented in chapters 2 and 5 of the dissertation, respectively. The following outline serves to assist the reader in finding key elements of the study in the dissertation:

- **Problem statement, study objectives and study layout**

  Chapter 1

- **Literature background**

  Chapter 2 (literature review), Chapter 3 (article introduction) and Chapter 4 (article introduction)

- **Materials and methods**

  Chapter 3 and 4 (materials and methods for the generation of data is presented in these articles) and Addendum A and C (additional materials and methods)

- **Results and discussion**

  Chapter 3 and 4 (results and discussion of studies presented in these articles) and Addendum B (additional results and discussion)

- **General discussion**

  Chapter 5 (general discussion of all findings in the study, i.e. findings presented in the articles (chapter 3 and 4) as well as from additional studies necessary to complete the study (i.e. in addenda)

- **Summary and conclusion**

  Chapter 5 (for the study as a whole, including findings presented in the articles and addendum B)

1.2 Problem statement

Methamphetamine (MA) is a drug abused worldwide by more than 35 million people and is a fast growing, serious problem (Vos *et al*., 2010). MA is cheap and easy to synthesize, making it the world's second most widely abused drug after cannabis (Vearrier *et al*., 2012). The drug has many desired (not necessarily beneficial) effects such as euphoria and increased energy levels, weight loss properties and a general sense of well-being (Vearrier *et al*., 2012). However, MA also presents with serious side-effects, including cognitive deficits, increased incidence of psychosis and also neurodevelopmental abnormalities (Kirkpatrick *et al*., 2012; Yamamoto *et al*., 2010).

Cause for concern is the development of psychosis in many MA abusing individuals. MA abusers are 11 times more likely to develop schizophrenia-like symptoms than the general population, termed MA psychosis (Vos *et al*., 2010). Studies indicate that chronic MA abuse can lead to the development of MA psychosis, manifesting as paranoid hallucinations, delusions and compulsive behaviour (Yui *et al*., 2000; Maxwell, 2005), although the exact mechanism by which MA induces psychosis is not known. Because MA-induced psychosis is so similar to paranoid schizophrenia, this study will focus on the development of psychotic symptoms and the possible mechanisms involved.

Schizophrenia is a neurodevelopmental disorder, linked to pre- and postnatal exposure to adverse environmental conditions such as early life stressors or toxins (Fone & Porkess, 2008). An alarming amount of women abuse MA during pregnancy, exposing the foetus to MA and its effects (Piper *et al*., 2011; Grace *et al*., 2010a). Studies have shown that children exposed to MA prenatally have distinct neurochemical and structural brain differences, especially in the striatum (Chang *et al*., 2007; Vearrier *et al*., 2012). Prenatal MA exposure could have serious effects on the development of the brain (McFadden *et al*., 2011) and could result in later life behavioural abnormalities that would include an increased susceptibility to psychotic events.

MA exerts its affect by influencing multiple neurotransmitter systems, the most important of which is the dopaminergic system. MA also affects serotonergic, noradrenergic and glutamatergic systems (Vearrier *et al*., 2012; Yamamoto & Raudensky, 2008). Subsequently MA can increase monoamines such as dopamine (DA), serotonin (5-HT) and noradrenalin (NA), ultimately resulting in oxidative stress seen in many MA users. Chronic abuse of MA results in the depletion of monoamines and can cause persistent cortico-striatal dopaminergic deficits in humans and animals (Imam & Ali, 2001; Grace *et al*., 2010b; Vearrier *et al* 2012). DA dysfunction is thought to play an important part in the behavioural deficits seen with MA abuse and may even be the primary mechanism involved in the drug's behavioural actions (Munzar & Goldberg, 2000). In this study I investigated the neurochemical changes seen after MA administration, especially focusing on monoamines (DA, 5-HT and NA) in the striatum and frontal cortex, as well as indications of oxidative stress (lipid peroxidation and superoxide dismutase activity) in these regions. Neurochemical changes may possibly correlate with psychosis seen in MA-abusing individuals.

In the current study I investigated the developmental effect of chronic (16-day) administration of MA or saline (vehicle control group) in Wistar rats subjected to pre- or postnatal exposure to MA and the presentation later in life of behaviours akin to schizophrenia in humans. In particular the effects on social interactive behaviours and sensorimotor gating was investigated, the latter determined by measuring prepulse inhibition (PPI) of the acoustic startle response. Wistar rats were either group-reared or social isolation reared (SIR) and I investigated the effect of the rearing conditions together with MA administration on the possible development of psychosis. SIR mimics the adverse environment that many MA abusers are subjected to and SIR together with MA administration may lead to an increased incidence of psychotic symptoms.

1.3 Study objectives

The primary objective of the current study was to establish whether chronic MA exposure during early life has adverse effects on neurodevelopment. More specifically, I aimed to establish which period of pre- or postnatal exposure to MA is more at risk for developing late-life abnormal behaviours related to schizophrenia in humans. Wistar rats were exposed to MA from prenatal day -13 to postnatal day +2 and a second group exposed to MA from postnatal days +35 to +50, the latter corresponding to early adolescence. Prenatal day -13 to postnatal day +2 was chosen to investigate the effects of MA exposure *in utero* on later life behaviour. Postnatal days +20 to +60 span puberty and it is during this period that MA abuse often begins. Thus postnatal days +35 to +50 were chosen to investigate the effect of MA abuse during the phase corresponding to human adolescence. This period could lead to better insight into the mechanisms involved in MA neurotoxicity, since the adolescent brain responds differently to drugs than either the mature or developing brain (Vorhees *et al*., 2005).

Since SIR is an established and well-validated neurodevelopmental model of schizophrenia (Möller *et al.,* 2011), it was also investigated whether early life exposure to MA (either pre- or postnatal) would bolster schizophrenia-like behaviours in a translational model of schizophrenia. Consequently group-reared animals and animals reared in isolation for 8 weeks (postnatal days +21 to +77) were exposed to either pre- or postnatal MA, as described above. The rats were tested in later adulthood (i.e. at the end of 8 weeks SIR on postnatal day +78) for altered sensorimotor gating and deficits in social behaviours.

The study specifically aimed to:

- Determine whether chronic early life postnatal exposure to MA may sensitize normal (group-reared) or predisposed (SIR) individuals to the development of schizophrenia-like behaviours later in life.
- Determine whether chronic prenatal (*in utero*) exposure to MA may sensitize normal (group-reared) or predisposed (SIR) individuals to the development of schizophrenia-like behaviours later in life.
- Determine schizophrenia like behaviour using the prepulse inhibition (PPI) and social interaction tests to determine deficits in sensorimotor gating and social- and self-directed behaviours, respectively.
- Investigate a possible correlation between the observed behavioural changes in the animals with respect to cortico-striatal brain monoamine levels and markers of oxidative stress.
- Compare group-reared rats to SIR rats in order to determine if prior adverse experience (SIR) represents a risk factor to developing more severe behavioural abnormalities following MA exposure.
- Investigate post-mortem changes in regional brain monoamines, superoxide dismutase activity (SOD) and lipid peroxidation in SIR vs. group-reared animals following either prenatal or postnatal MA exposure.

1.4 Study layout

Due to renovations and improvements undertaken at the Animal Centre at the North-West University, Potchefstroom, all experiments for the current study were performed at the National Health Laboratory Services, Edenvale, South Africa. For the above mentioned study objectives to be achieved the following study layout was developed:

- Animals: Adult male Wistar rats, all weighing 250 g – 300 g on the day of behavioural testing, were used for the study. 2 groups of rats were used in this study, viz. grouped-reared Wistar rats and SIR Wistar rats.

- These 2 groups were further divided into prenatal MA exposure groups and postnatal MA exposure groups (see Fig. 1-1), where half of these rats received MA injections subcutaneously (s.c.) and the other half received saline injections (Fig. 1-1). The prenatal group was exposed to MA from days -13 to +2 via dosing of the pregnant dams, while the postnatal group (weaned pups) received MA from days +35 to 50. In the prenatal group six rats per group were used (n=6) and in the postnatal group ten rats per group were uses (n=10).

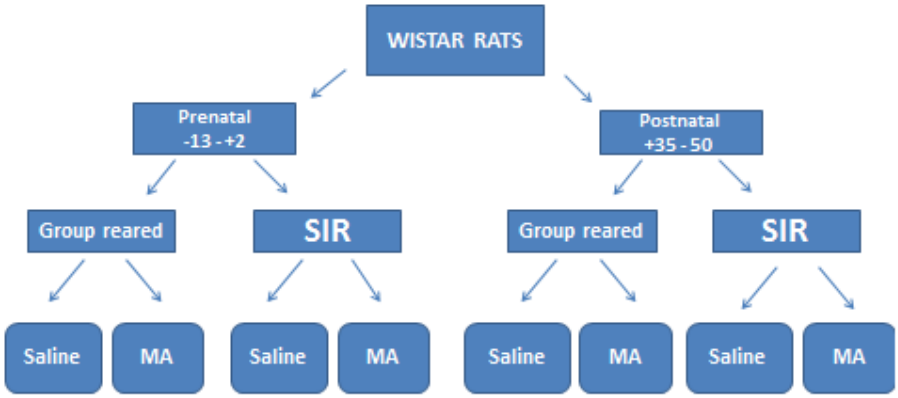

Figure 1-1: **Study layout for the pre- and postnatal groups. SIR – Social isolation-reared, MA - Methamphetamine.**

- Drug treatment: For the prenatal study, dams were injected from gestational day -13 to +2 with 5 mg/kg MA administered s.c. once daily. For the postnatal study, rats were injected from days +35 to +50 with an escalating dosage regimen of 0.2 mg/kg ending at 6 mg/kg s.c. twice a day.
- SIR: In the SIR group, rats were reared in isolation from postnatal day +21 to +77 (8 weeks), whereas the concurrent control groups were group-reared.
- Behavioural Testing: Behavioural testing started at the end of the 8 week long group-rearing or SIR period rearing, whereupon PPI and social interaction was assessed on postnatal day +78.
- Neurochemical Testing: 24 hours after behavioural testing, rats were sacrificed and brain tissue collected for later neurochemical analysis.

1.5 Expected results

The working hypothesis for this study is that chronic MA administration will induce schizophrenia-like behaviour and associated neurochemical changes, while combining MA with a population at risk, viz. SIR, will lead to a worsening of these changes. Also I hypothesize that prenatal exposure to MA will result in a greater predisposition to develop psychosis later in life.

This work will shed new light on the complex neurobiology of MA abuse and its subsequent effects on behaviour and neurochemistry.

Chapter 2 – Literature review

2.1 Introduction to methamphetamine

Methamphetamine (MA) is an analog of amphetamine and produces similar psychostimulant effects, including increased euphoria, sociability and alertness (Kirkpatrick *et al*., 2012). Because of its strong euphoric properties, this drug is widely abused around the world, making it a serious and growing problem (Yamamoto *et al*., 2010). A study by Vos *et al* (2010) indicates that more than 35 million people around the world abuse MA on a regular basis. The drug is mainly abused for its desirable effects on energy levels, wakefulness and the sense of well-being that it imparts (Vearrier *et al*., 2012; Herring *et al*., 2008). Many people, including students, truck drivers and professionals, have used MA to stay awake and/or to improve attention (Vearrier *et al*., 2012), whereas it is also abused for its weight loss potential (Nyabadza & Hove-Musekwa, 2010). Moreover, MA is easy and cheap to synthesize, and also its euphoric effects last longer than that of cocaine, rendering it very popular (Heller *et al*., 2001a). Long-term MA abuse has a significant impact on the economy, where drug abuse is estimated to cost around 1 trillion dollars per year worldwide (Thrash *et al*., 2009).

Amphetamines, including MA, were first developed as synthetic alternatives to ephedra, an extract of *Ephedra sinica, a* plant that was originally used in traditional Chinese medicine. Ephedrine was extracted from ephedra in 1885 and it was soon discovered to have similar actions to epinephrine. Amphetamine-type stimulants were subsequently developed in the search for a synthetic substitute for ephedrine (Vearrier *et al*., 2012).

MA was first synthesized in 1919 by a Japanese chemist, Akira Ogata, using ephedrine as a precursor. Because of its vasoconstrictive and bronchodilatory properties, it gained popularity in health management and by 1932 MA was marketed for use in the treatment of asthma and nasal congestion. Since then, MA has had many different indications, including some off-label uses. To this end, MA was subsequently used as an appetite suppressant, to treat morphine or codeine addiction, as well as alcoholism, migraine, myasthenia gravis, Meniere's disease, epilepsy and even seasickness (Vearrier *et al*., 2012). MA also played a prominent role in World War II where it enhanced the mental power and fighting spirits of soldiers (Yui *et al*., 2000), and countries such as Germany, Japan and the USA used MA to increase alertness and wakefulness and to suppress the appetite of soldiers. The drug was quickly being abused and restrictions were placed on its use (Vearrier *et al*., 2012). In the USA, MA is approved by the FDA to treat attention deficit disorder as well as weight loss in women, although the use of MA in these conditions is ill-advised (Kast, 2010).

2.1.1 Epidemiology of MA as street drug

MA is mostly abused by adolescents between the ages of 14 and 18 years (Kokoshka *et al*., 2000) and most abusers tend to be single and unemployed (Vos *et al*., 2010). These young abusers also tend to live in rural rather than urban regions (Grant *et al.,* 2011). Of growing concern is evidence that drug abuse during these early years may result in long-term psychiatric and neurological problems (Kokoshka *et al*., 2000). In the Cape Town region in South Africa, the abuse of MA has lately seen market increases (Vos *et al*., 2010). Compared to other provinces in South Africa, the Western Cape Province is the most affected by drugs such as MA (Nyabadza & Hove-Musekwa, 2010). Here, MA is referred to in the colloquial as "Tik", which is derived from the clicking/crackling sound the drug makes when it is being heated (Plüddemann *et al*., 2008). Other street names for MA include "Meth", "Speed" and "Crank". MA is highly addictive (Vos *et al*., 2010) and is usually smoked in a crystalline form, known as "crystal meth" or "Ice", which is regarded as more potent (Plüddemann *et al*., 2008). This form of MA is relatively pure (purity levels above 80%) and its physical effects are known to have a longer duration (Maxwell, 2005). The crystalized form is very easy to synthesize from cheap, readily available, house-hold chemicals (Vos *et al*., 2010).

Most drugs of abuse, like cannabis and cocaine, originate from natural resources. MA, on the other hand, is prepared synthetically (Thrash *et al*., 2009). Since ephedrine and pseudoephedrine are readily accessible drugs, being bought over the counter as medicines for the symptomatic relief of common cold, they have been used as precursor chemicals to manufacture MA. Other chemicals used in the illicit manufacturing of MA include household items like paint thinner, fertilizer, alcohol, table salt, drain cleaners, electrochemical batteries and lighter fluid (Vearrier *et al*., 2012).

MA can be administered via different routes, for instance orally, inhaled (smoked) and intravenous injection (Kirkpatrick *et al*., 2012) or it can be administered as an anal suppository (Vearrier *et al*., 2012). Dosing patterns can vary between users, although typical patterns of abuse have emerged. Many users go on binges lasting around 4 days, with each binging episode consisting of 4 or more daily doses of MA. Individual doses can range between 50 mg and 500 mg and can amount to as much as 4 g daily (Cruickshank & Dyer, 2009).

Drug users typically abuse more than one drug, so that MA abuse has been associated with the simultaneous (co-) abuse of cannabis, alcohol, heroin and opioids. These substances can increase the toxicity of MA, rendering the combinations potentially lethal at lower doses than observed with the respective drugs alone. When alcohol is combined with MA, the abuser can experience tachycardia and hypertension to a much greater degree than with MA use alone. The combination of MA and heroin, as well as the combination of MA and cocaine has been described to exacerbate cardiotoxic effects (Darke *et al*., 2008).

Chronic abusers of MA are 11 times more likely to develop psychotic symptoms (i.e. MA-induced psychosis) than the general population (Vos *et al*., 2010). Because of this close association, it is important that psychosis and schizophrenia are briefly reviewed before MA is studied at a deeper level.

2.2 The neurobiology of schizophrenia

Schizophrenia is one of the most devastating psychiatric disorders, affecting around 1% of the world's population (Nilsson *et al*., 2005). The symptoms of this heterogeneous disease are mainly divided into 3 categories: positive symptoms, negative symptoms and cognitive deficits (Bitanihirwe & Woo, 2011). The positive symptoms include hallucinations and delusions, whereas negative symptoms usually include a decrease in energy levels and interest, reduced apathy and production of speech, reduced emotional expression and reaction, and diminished involvement in interpersonal relationships. Cognitive deficits such as learning and memory deficits also play an important part in schizophrenia, resulting in an impairment of skills and reduced functional capacity (Ross *et al*., 2006).

Many factors have been hypothesized to contribute to the development of schizophrenia, of which early-life neurodevelopmental damage is one of the foremost factors thought to contribute to the development of the disorder (Daenen *et al*., 2003). Such neurodevelopmental changes can originate from foetal infections (viral or bacterial), childhood trauma, isolation, psychosocial stress, smoking and certain gene mutations, making the person more vulnerable to the development of schizophrenia (Seeman, 2011). Schizophrenia is also a highly heritable disease, with individuals more likely to develop the disease if a close family member has the illness (Ross *et al*., 2006).

Although the exact neurochemistry involved in schizophrenia is not known, there are several hypotheses regarding the aetiology of the disease. The first hypothesis involves the disruption of dopamine (DA) transmission. This hypothesis is based on the fact that amphetamines like MA can induce a psychotic-like state similar to schizophrenia, presumably by increasing DA release in the striatum, especially the nucleus accumbens, and representing the positive symptoms of the illness (Leonard, 2003). Indeed the DA hypothesis has been central to our understanding of the neurobiological mechanisms of the illness, especially since dopaminergic D_2 receptor blockade remains a necessary and sufficient component for antipsychotic action (Kapur & Mamo, 2003). On the other hand, deficits in DA neurotransmission in the frontal cortex has been linked to the cognitive and negative symptoms of schizophrenia (Wong & Van Tol, 2003; Ross *et al.,* 2006), as has deficits in glutamatergic neurotransmission in the cortex (Reynolds, 2008; Nilsson *et al*., 2005).

Patients suffering from schizophrenia show decreased glutamate (GLU) in the brain (Nilsson *et al*., 2005). The “GLU hypofunction” theory of schizophrenia was developed from the observation that inhibition of glutamatergic N-methyl-D-aspartate (NMDA) receptors, with for example phencyclidine or ketamine, induces an array of schizophrenia-like behaviours in humans (Zuo *et al*., 2012). Cortical GLU hypofunction results in ineffective activation of γ-amino butyric acid (GABA) inhibitory neurons, which in turn culminates in a reactive disinhibition of subcortical GLU neurons (Carlsson *et al*., 2001) and antagonism of the NMDA receptor. Subsequent overt elevation of intracellular Ca^{2+} concentrations will ultimately lead to neuronal damage (see § 2.3.5.1) (Stone, 2001). Since this response has been found to be directly related to increased GLU and DA release in brain regions involved in schizophrenia, viz. the medial prefrontal cortex and nucleus accumbens (Zuo *et al*., 2012), it argues that schizophrenia involves a combined state of GLU *and* DA dysfunction. Serotonin (5-HT) also plays a role in the development of the disease, where specifically a decrease in 5-HT has been linked to the depressive behaviour seen in schizophrenia (Reynolds, 2008; Möller *et al*., 2012), whereas excessive frontal cortical 5-HT is believed to underlie frontal lobe DA dysfunction (Leonard, 2003). Apart from evidence for the involvement of diverse neurotransmitters in the disorder, patients suffering from schizophrenia also show an increase in oxidative stress (Bitanihirwe & Woo, 2011), a manifestation that has recently been replicated in a neurodevelopmental animal model of schizophrenia (Möller *et al*., 2011).

Evidence for the involvement of GLU and oxidative stress in schizophrenia has directed researchers to consider another endogenous pathway linked to GLU dysfunction, oxidative stress and inflammation, namely the kynurenine pathway. Tryptophan is catabolized via the kynurenine pathway to either kynurenic acid (KYNA) or to 3-hydroxyanthranilic acid (3-OHAA) and quinolinic acid (QA) (see Fig. 2-1) (Schwarcz, 2004; Stone, 2001). KYNA has antagonistic properties on NMDA receptors in the brain with potential neuroprotective properties (Olsson *et al*., 2012; Nilsson *et al*., 2005). 3-OHAA on the other hand is involved in the generation of free radicals, whereas QA is a NMDA receptor agonist and excitotoxin (Schwarcz, 2004; Stone, 2001). Therefore, alterations in equilibrium between QA and KYNA can result in oxidative stress and neuronal damage. Schizophrenia is also associated with altered release of pro- and anti-inflammatory cytokines (Drzyzga *et al*., 2006) that, via the effect of these immune modulators on indoleamine-2,3-dioxygenase (IDO) (Fig. 2-1), can directly affect kynurenine metabolism and as such destabilize GLU homeostasis (Myint *et al*., 2007). Consequently the kynurenine pathway is a major contributor to redox potential and neuroprotective:neurotoxic balance in the cell (Möller *et al.,* 2012). Elevated levels of QA can be observed in various brain regions of schizophrenic patients (Stone, 2001) and has also been demonstrated in social isolation reared (SIR) rats, a putative neurodevelopmental animal model of schizophrenia (Möller *et al*., 2012). Studies have shown that KYNA controls glutamatergic neurotransmission and an increase in KYNA has been

linked to the hyperdopaminergic activity seen in patients suffering from schizophrenia (Nilsson *et al*., 2005).

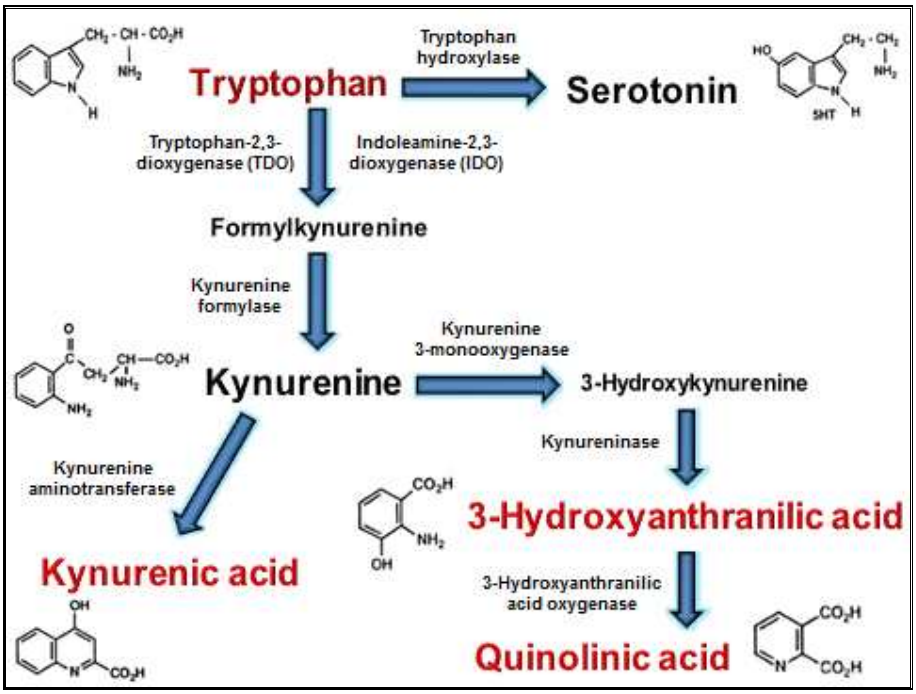

Figure 2-1: **Kynurenine pathway of tryptophan metabolism and the enzymes involved. Tryptophan is metabolized to kynurenic acid, 3-hydroxyanthranillic acid and quinolinic acid. In the liver tryptophan is metabolized via tryptophan-2,3-dioxygenase to kynurenine and in most other tissue tryptophan is metabolized via indoleamine-2,3-dioxygenase (Adapted from Stone, 2001)**

Within the context of this study, abnormal DA levels can be seen in many neurological disorders, including schizophrenia and depression, and invariably may be linked to DA transporter (DAT) dysregulation. This is of great concern, since many psychostimulant drugs such as MA exert their action on the DAT, resulting in disrupted DA levels or dopaminergic neurodegeneration (Cervinski *et al*., 2005). Studies have also demonstrated that amphetamine administration can reduce the levels of KYNA in the striatum of young rats, possibly contributing to neurotoxicity (Stone, 2001).

Drug treatments currently available to treat schizophrenia are only partially successful and far from optimal. About 20% of schizophrenia patients, regardless of treatment, relapse within a year (Harvey *et al*., 1999). Antipsychotics are generally used to treat the disorder. First generation antipsychotics including haloperidol and chlorpromazine are effective in reducing the positive symptoms of schizophrenia, exerting their primary action by blocking the dopaminergic D_2 receptor. Second generation antipsychotics like olanzapine and clozapine block the dopaminergic D_2 as well as the serotonergic 5-HT_{2A} receptors and are preferred because of their lower side effect profile (Ross *et al*., 2006; Geyer *et al*., 2012). Despite advances in our knowledge of schizophrenia, and the many new generation antipsychotic agents now available, clinical efficacy has not improved that significantly, especially with regard to cognitive and

negative symptoms (Geyer *et al*., 2012). It is evident that a better understanding of the disorder is needed in order to improve treatment outcomes (Ross *et al*., 2006).

2.3 Methamphetamine

2.3.1 Adverse effects of MA abuse

2.3.1.1 Neurological and psychiatric effects

MA abuse can lead to long-term effects such as cognitive impairments and psychological problems (Kirkpatrick *et al*., 2012). Cognitive deficits include impaired performance on memory tests, lower attention span, impaired abstract thinking and ability to manipulate information (Vearrier *et al*., 2012), and difficulties in decision making (Grant *et al*., 2011). The most important neurological adverse effect is intracranial hemorrhage, associated with MA-induced hypertension and tachycardia. The abuse of MA has also been linked to ischemic stroke (Vearrier *et al*., 2012).

MA is commonly associated with psychosis, with hallucinations and delusions presenting as consistent symptoms (Grant *et al*., 2011). This psychotic state can manifest as paranoid hallucinations, delusions and compulsive behaviour (Yui *et al*., 2000; Maxwell, 2005) and is indistinguishable from acute or chronic paranoid schizophrenia. Other adverse psychiatric effects include irritability and agitation that can leave the patient confused and in a panicked state (Thrash *et al*., 2009). The psychotic state associated with MA abuse can persist even after the pharmacological effects of the drug have worn off. Moreover, MA psychosis can spontaneously reoccur, especially in response to stressful situations, including physical or psychological stress (Yui *et al*., 2000). In some cases, MA-induced psychosis can precipitate violent behaviour (Darke *et al*., 2008). Importantly, MA can exacerbate or induce psychosis in persons already suffering from schizophrenia or produce symptoms in people with no previous history of psychiatric illness (Grant *et al*., 2011). The mechanism(s) for this cross-sensitivity may involve various common elements evident in schizophrenia and MA psychosis, one example being DA and redox dysfunction, as will be discussed in due course below.

Mood- and anxiety disorders have also been documented, where many MA abusers experience irritability, hyperawareness, depression, agitation, impulsivity and even violent behaviour (Vos *et al*., 2010; Maxwell, 2005). Other stimulant effects include an increase in heart rate and blood pressure, sleep disruptions, decreased food intake (Kirkpatrick *et al*., 2012; Maxwell, 2005), dilated pupils (Cruickshank & Dyer, 2009) and an increase in locomotor activity, hyperthermia, and aggressiveness (Yamamoto *et al*., 2010).

2.3.1.2 Oral, dermatological and other effects

Tooth decay, better known as “meth mouth”, is due to suppression of saliva secretion and is a common adverse effect of MA abuse. MA abuse results in xerostomia, caused by sympathetic overstimulation, while many abusers indulge in grinding or chewing movements, referred to as bruxism (Vearrier *et al*., 2012; Maxwell, 2005). These mechanisms may then result in dental decay. MA aside, drug abuse in general is associated with a lack of oral hygiene and malnutrition (Vearrier *et al*., 2012).

MA abusers may be at a greater risk of developing methicillin resistant *Staphylococcus aureus* infections, mostly associated with formication. Formication is where a MA abuser experiences a sensation of something crawling on the skin and then picks at the skin, causing damage to the skin and thus increasing vulnerability to infection (Cohen *et al*., 2007; Cruickshank & Dyer, 2009). As a further manifestation of hyperdopaminergia, MA abuse has been associated with stereotypical and repetitive skin-picking (Vearrier *et al*., 2012) that also contributes to dermatological problems.

2.3.1.3 Cardiovascular and hepatic effects

Repeated administration of MA may result in chronic cardiac conditions like cardiomyopathy or coronary heart disease (Cruickshank & Dyer, 2009; Maxwell, 2005). Cardiomyopathy may be caused by a variety of mechanisms, including diffuse myocardial toxicity caused by an overstimulation of cardiac noradrenergic receptors and coronary artery spasm (Vearrier *et al.,* 2012).

Acute liver injury has also been reported with MA abuse, resulting in hepatic necrosis. MA may also enhance the hepatic toxicity of substances like carbon tetrachloride (Vearrier *et al*., 2012).

2.3.1.4 MA associated genitourinary and haematological effects

Cause for concern is the effect the drug has on sexual behaviour. MA has been known to enhance sexual desire (Plüddemann *et al*., 2008) and this can lead to high risk sexual behaviour (Vos *et al*., 2010; Maxwell, 2005). Considering the high prevalence of human immunodeficiency virus (HIV) in South Africa, the increased likelihood of unplanned, unsafe sex with multiple partners is of great concern (Plüddemann *et al*., 2008). MA abuse is the highest in the Western Cape Province of South Africa, while this province also has the highest rise in new HIV infections (Nyabadza & Hove-Musekwa, 2010). In accordance with this, studies have shown that MA can increase the risk of contracting HIV (Plüddemann *et al*., 2008; Maxwell, 2005) and also other sexually transmitted diseases (STD’s) (Vearrier *et al*., 2012). Because of their risky sexual behaviour and the sharing of contaminated needles, MA abusers also have an associated higher risk of contracting viral hepatitis A and B. Hepatitis C infection is also common among abusers using contaminated needles (Vearrier *et al*., 2012).

2.3.1.5 MA overdose

MA overdose can present with a variety of symptoms. The patient may experience rapid respiration and extreme agitation, with tachycardia, hyperthermia (Cruickshank & Dyer, 2009) and excited delirium (Darke *et al*., 2008). Dilated pupils and shivering is also common and an overdose can cause cardiac, renal or hepatic failure (Cruickshank & Dyer, 2009). MA overdose can result in nausea, vomiting and even seizures (Darke *et al*., 2008). An altered mental state may result in psychosis and/or agitation (Cruickshank & Dyer, 2009) and the patient may experience panic, hallucinations and paranoia (Darke *et al*., 2008).

2.3.1.6 MA dependence and treatment

MA abusers usually develop dependence on the drug. With repeated use, the abuser can develop tolerance for MA, resulting in the use of higher doses, more potent forms of MA, and other routes of administration in order to get the desired response (Darke *et al*., 2008). MA withdrawal can occur with abrupt cessation of the drug, with the severity of withdrawal depending on the dosage and duration of drug abuse. Withdrawal symptoms may include depression, agitation, anxiety and unease, cravings for MA, sleep disruptions with unpleasant dreams, excessive eating, reduced energy (Cruickshank & Dyer, 2009; Srisurapanont *et al.,* 1999), irritability, decreased concentration (Maxwell, 2005) and anhedonia (Thrash *et al*., 2009). Some people experience persistent depression after the cessation of MA, indicating the possible development of neurotoxicity (Cruickshank & Dyer, 2009).

There is no specific treatment for MA abuse and usually the same approach as with cocaine abuse is taken. To treat drug addiction, pharmacological and behavioural interventions are needed. Behavioural and psychosocial interventions focus on an attempt to decrease the reward response associated with drug abuse, thereby to dampen the drug-seeking behaviour (Maxwell, 2005, Thrash *et al*., 2009). Aversion therapy, where MA abuse is paired with an unpleasant stimulus (e.g. chemical or electrical), has also been suggested to have promising results (Cretzmeyer *et al*., 2003). A protocol for contingency management might also play an important role in the treatment of MA abuse. This includes drug counselling and drug testing to prevent relapse and to help manage cravings (Marinelli-Casey *et al.,* 2008; Cretzmeyer *et al*., 2003). Medication may also play an important role, with replacement strategies similar to that used in opioid or nicotine addiction (Maxwell, 2005). Antipsychotics can also be helpful in managing MA-induced psychosis (Cretzmeyer *et al*., 2003).

2.3.2 Chemistry & physical properties of MA

The structure of MA (Fig. 2-2A) is similar to amphetamine and methcathinone (Fig. 2-2B & C), both being produced through the chemical reduction of pseudoephedrine or ephedrine (Thrash *et al*., 2009). MA is the *N*-methyl derivate of amphetamine (Fig. 2-2B; Cruickshank & Dyer,

2009) and falls under the phenylethylamine class of psychostimulants, others of which include amphetamine, ephedrine, pseudoephedrine and methylenedioxymethamphetamine (MDMA). MA is known to have a more rapid onset of action, due to its rapid distribution in the central nervous system (CNS). This rapid action can be ascribed to the added N-methyl group, making the drug more lipid soluble and thus enhancing brain penetration across the blood-brain barrier (BBB; Vearrier *et al*., 2012, Thrash *et al*., 2009). These lipophilic properties also increase the drug's resistance to degradation by monoamine oxidase (MAO; Thrash *et al*., 2009). The desired euphoria is reached more rapidly than with other amphetamines (Vearrier *et al*., 2012).

MA consists of a mixture of *d*- and *l*-stereoisomers. The *d*-stereoisomer of MA is up to 10 times more potent at causing DAT disruption and thus inhibiting DA reuptake (see Fig. 2-4, § 2.3.4.1). This stereoisomer is also responsible for the euphoria associated with MA, whereas *l*-MA possesses little CNS effects (Vearrier *et al*., 2012; Volz *et al*., 2007). The crystalline form of MA (*d*-methamphetamine hydrochloride), known as "ice" or "crystal meth", is white or translucent crystals, used for inhalation (Cruickshank & Dyer, 2009).

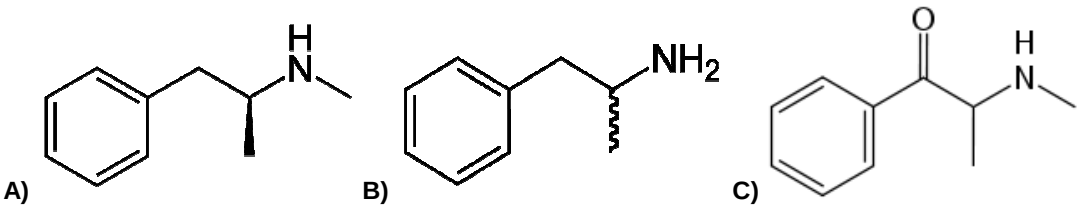

Figure 2-2: **(A) Chemical structure of MA – $C_{10}H_{15}N$; (B) Chemical structure of amphetamine – $C_9H_{13}N$; (C) Chemical structure of methcathinone – $C_{10}H_{13}NO$**

2.3.3 Pharmacokinetics of MA

MA is metabolized in the liver via 3 different routes. Firstly, MA is metabolized by *N*-demethylation, producing amphetamine by an enzymatic process catalyzed by cytochrome P450 2D6. Secondly, MA is metabolized by aromatic hydroxylation to produce 4-hydroxymethamphetamine, a process that also involves cytochrome P450 2D6. Lastly, MA undergoes β-hydroxylation to produce noradrenalin (NA; Cruickshank & Dyer, 2009). MA is mostly excreted in the urine and the elimination half-life is approximately 10-12 hours (Cruickshank & Dyer, 2009; Herring *et al*., 2008).

2.3.4 The pharmacodynamics of MA

2.3.4.1 The synaptic actions of MA and its effects on monoamines

MA impacts on multiple neurotransmitter systems, especially DA neurotransmission. However, the drug also affects NA, 5-HT and GLU neurotransmission (Vearrier *et al*., 2012; Yamamoto & Raudensky, 2008). The structural homology between MA and endogenous monoamines such as DA (see Fig. 2-3), NA and 5-HT, allows MA to substitute for these monoamines at their

membrane-bound transporters. These transporters include the DAT, NA transporter (NAT), 5-HT transporter (SERT) and vesicular monoamine transporter-2 (VMAT-2; Cruickshank & Dyer, 2009). These transporters regulate monoamine concentrations in the cytoplasm through transport back into nerve terminals.

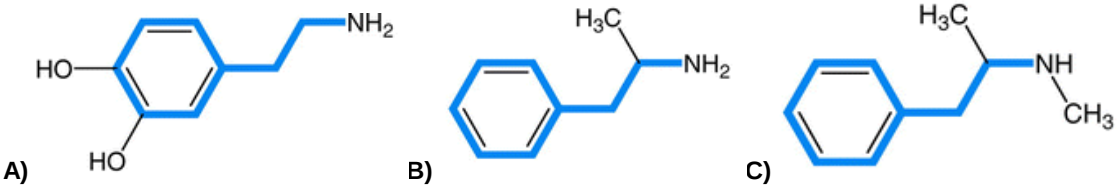

Figure 2-3: **Structural similarities between DA (A), amphetamine (B) and MA (C).**

MA is a potent releaser of DA, NE (Kirkpatrick *et al*., 2012) and 5-HT (Yamamoto *et al*., 2010), and acts as an indirect agonist at these receptors (Cruickshank & Dyer, 2009). The mechanisms whereby MA modifies the release of monoamines will be discussed in more detail further below, but can be summarized as follows (see Fig. 2-4):

- MA causes a reverse transport of monoamines, causing them to move from the cytoplasm into the synaptic cleft as seen in Figure 2-4. Amphetamines are known to be substrates for DA, NE and 5-HT transporters and can thus be taken up by the nerve terminal via these transporters and cause reverse transport of the associated biogenic amines. They can also diffuse into the terminals (Yamamoto *et al*., 2010; Sulzer *et al*., 1995).
- MA reverses the function of VMAT-2 (see Fig. 2-4) and disrupts the pH gradient, causing the monoamines to be redistributed (Cruickshank & Dyer, 2009). VMAT-2 is relocated from its synaptosomal location to a nonsynaptosomal location, inhibiting its ability to sequester DA into synaptic vesicles for storage and later release (Vearrier *et al*., 2012; Riddle *et al*., 2002).
- Increased DA and 5-HT release from storage vesicles and prevention of uptake into the vesicles. This increases the concentration of DA and 5-HT in the cytoplasm and more of these neurotransmitters can be made available for reverse transport (Fig. 2-4; Yamamoto *et al.,* 2010).
- In the synaptic cleft, the reuptake of monoamines are inhibited as shown in Figure 2-4 (Yamamoto *et al*., 2010), thereby increasing the availability of transmitter to bind to pre- and post-synaptic receptors.
- MA inhibits MAO responsible for monoamine metabolism, further increasing the levels of DA, NA and 5-HT (Cruickshank & Dyer, 2009).

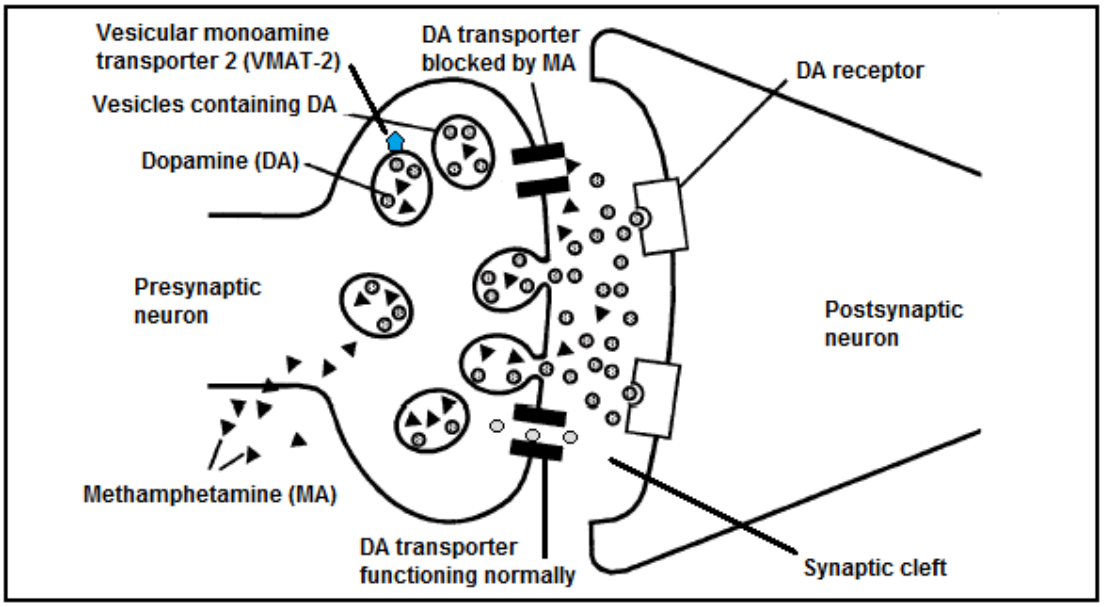

Figure 2-4: **MA mechanism of action – effects on DA. MA increases the release of DA and blocks the DA transporter, resulting in more DA in the synaptic cleft (Adapted from Sasek, 2005)**

The activation of DA D_1 and D_2 receptors plays a prominent role in MA-induced deficits in dopaminergic function. These receptors are also involved in GLU signaling, reactive oxygen species (ROS) formation and DAT and VMAT-2 dysfunction (Riddle *et al*., 2006), all of which play a distinct role in the neurotoxic profile of MA. This implies that MA will have an effect on 2 major dopaminergic pathways in the brain, namely the nigrostriatal and mesolimbic pathways, shown in Figure 2-5 (Thrash *et al*., 2009).

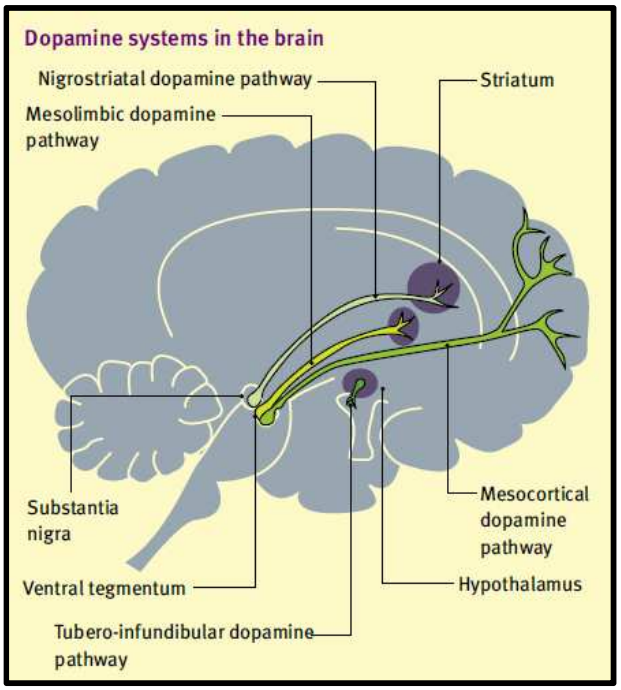

Figure 2-5: **DA pathways in the brain (Reynolds, 2008)**

Studies have shown that tyrosine hydroxylase (TH) activity may also decrease due to MA abuse (Imam & Ali, 2001; Grace *et al*., 2010b). Along with that, MA can also cause a decrease in TH (McCann & Ricaurte, 2004) and a decrease in neuronal density (Cruickshank & Dyer, 2009), as well as many transporters such as the DA, 5-HT and VMAT-2 transporters (McCann & Ricaurte, 2004; Grace *et al*., 2010b). VMAT-2 transports DA into synaptic vesicles for later synaptic release (Fig. 2-4). Dysfunction of the VMAT-2 in striatal synaptic terminals caused by MA results in more DA being available for auto-oxidation, thereby damaging nerve terminals (Riddle *et al*., 2006; Riddle *et al*., 2002). Changes in these transporters correspond with memory and cognitive impairments, with difficulty in manipulation of information and attention (Grace *et al*., 2010b).

When used chronically, MA causes long-term depletion of monoamines such as DA and its metabolites (Imam & Ali, 2001; Grace *et al.,* 2010b), so that repeated administration of high dose MA causes persistent striato-limbic dopaminergic deficits in both animals and humans (Vearrier *et al*., 2012). In the striatum (Fig. 2-6), MA decreases the amount of DAT (Imam & Ali, 2001; Grace *et al*., 2010b), whereas effects on DA terminals in this region is also affected more markedly than in other regions (Yamamoto *et al*., 2010). MA administration results in a reversal of DAT function by internalization of DAT (Cervinski *et al*., 2005; Vearrier *et al*., 2012), thereby increasing extracellular DA levels (Riddle *et al*., 2006). This in turn impairs DAT, and the resulting inhibition of DA reuptake promotes the oxidation of DA and the formation of ROS (Vearrier *et al*., 2012; Riddle *et al*., 2006). 5-HT terminals, on the other hand, are equally sensitive to the damaging effects of MA in all brain areas (Yamamoto *et al*., 2010), but particularly the hippocampus (see Fig. 2-6; Kuczenski *et al*., 2007). Brain areas other than the striatum, which are also sensitive to the effects of MA, include the prefrontal cortex (shown in Fig. 2-6) and amygdala (Yamamoto *et al*., 2010). Changes observed in the medial prefrontal cortex after MA administration has been linked to the development of behavioural sensitization and subsequent MA-induced psychosis (Kadota & Kadota, 2004).

All the effects of MA can be explained by possible monoaminergic mechanisms involving DA, NA and 5-HT. The medial basal forebrain is part of a noradrenergic region and when stimulated can mediate arousal. Noradrenergic stimulation in the prefrontal cortex is involved in processing cognitive function (Cruickshank & Dyer, 2009). Euphoria and excitement are typical responses to MA abuse, and is due to stimulation of the mesolimbic reward system (Thrash *et al*., 2009). Excessive DA activation in the striatum can result in paranoia and hallucinations. The stimulation of 5-HT receptors (5-HT_1 and 5-HT_{2B}) can have an anxiolytic effect and can make people more sociable and result in self-confidence. 5-HT neurons also regulate sexual behaviour, hyperthermia, respiration and anxiety (Cruickshank & Dyer, 2009). 5-HT over-activation is also responsible for hypodopaminergia in the frontal cortex resulting in negative symptoms (Harvey *et al*., 1999).

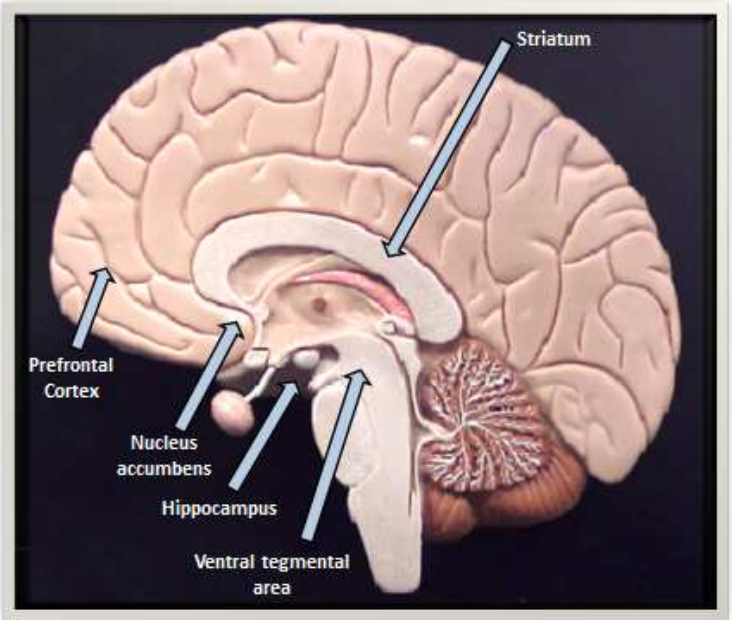

Figure 2-6: **Brain areas affected MA (Adapted from Volkow *et al.*, 2001a; Volkow *et al.*, 2001b)**

It is thus clear from the above that MA results in dopaminergic neurotoxicity, thought to be caused by the generation of free radicals, such as ROS and reactive nitrogen species (RNS; Imam & Ali, 2001; Riddle *et al*., 2006). Many studies have reported this potentially neurotoxic effect of MA, especially towards DA neurons (McCann & Ricaurte, 2004; Frost & Cadet, 2000). Although neurotoxicity is evident following MA abuse, the exact process is not known and many different mechanisms have been identified that may contribute to the neuronal damage caused by MA. Neurotoxicity is evidenced by the appearance of long-term striatal DA depletion together with degeneration of DA terminals, as well as a decrease in DAT and SERT (Tata & Yamamoto, 2007).

Yamamoto *et al* (2010) have highlighted a number of known and emerging mechanisms that might cause neurotoxicity or be a result of the neurotoxic effects of MA. This latter study divides the aspects of toxicity into 2 categories, namely classical aspects and new and emerging aspects. Classical aspects that may lead to MA toxicity include oxidative stress, excitotoxicity, mitochondrial dysfunction and hyperthermia. After MA is administered, there is an increase in intra- and extracellular DA, as well as an increase in extracellular GLU together with hyperthermia. When this phenomenon occurs in combination with oxidative stress, excitotoxicity and mitochondrial dysfunction, long term negative effects emerge. New and emerging signs of MA-associated neurotoxicity include excitotoxicity to nonmonoaminergic cell bodies, the presence of apoptotic proteins and activation of the ubiquitin proteasomal system, inflammatory cytokines, and BBB dysfunction. In addition, HIV and environmental stress have also been suggested as new and emerging manifestations that might contribute to MA-associated neurotoxicity. Not only is MA damaging to DA and 5-HT terminals, but endothelial and neuronal

cell bodies are also destroyed (Yamamoto *et al*., 2010). The following section provides a more detailed analysis of how MA abuse impacts on the central nervous system.

2.3.5 The Neurobiological basis of MA toxicity

2.3.5.1 MA and oxidative stress

The cytotoxic effects caused by ROS can be defined as oxidative stress. When ROS are increased they can lead to deoxyribonucleic acid (DNA), protein and lipid damage, resulting in protein nitration or lipid peroxidation, respectively (Tata & Yamamoto, 2007).

MA-related oxidative stress has been shown in many previous studies. MA-associated production of ROS and RNS is accompanied by the accumulation of lipid peroxidation products as shown in Fig. 2-7 (Yamamoto *et al*., 2010), associated with an increase in malonyldialdehyde in the hippocampus and striatum (Tata & Yamamoto, 2007). Some reactive species are generated through microglial activation and studies show that MA administration activates microglia in the striatum (see Fig. 2-6 – areas affected by MA; Riddle *et al*., 2006; Thomas *et al*., 2004). Microglial cells are antigen-presenting cells that secrete factors after activation, leading to neuronal damage (shown in Fig. 2-7). These factors include prostaglandins, nitric oxide (NO) and superoxide (Thomas *et al*., 2004). This implies that MA administration can result in inflammation, possibly contributing to neurotoxicity. ROS include superoxide radicals, hydrogen peroxide, hydroxyl radicals (Frost & Cadet, 2000) and semiquinones (Thrash *et al*., 2009), whereas RNS include peroxynitrite (Brown & Yamamoto, 2003). Redox cycling oxidizes DA into quinones and semiquinones, which can later convert into radicals such as nitrogen and superoxide radicals. This in turn can exert oxidative stress (Thrash *et al*., 2009). Not only are free radicals increased, but the levels of critical antioxidants in the DA and 5-HT terminals (Yamamoto *et al.,* 2010), such as glutathione peroxidase, superoxide dismutase, catalase and uric acid (Mirecki *et al*., 2004; Yamamoto & Raudensky, 2008), are also decreased, thereby making the terminals vulnerable to oxidative damage.

As mentioned earlier, MA abuse leads to an increase in DA (Yamamoto *et al*., 2010), resulting in a subsequent increase of toxic redox intermediates. The latter results from the enhanced autoxidation of DA (see Fig. 2-7; Brown *et al*., 2003; Frost & Cadet, 2000), the like of which include free radicals, quinones (Yamamoto *et al*., 2010), peroxynitrite, arachidonic acid and NO (Brown *et al*., 2003). Oxidized metabolites of monoamines include 6-hydroxydopamine, an oxidation product of DA, and 5,6-dihydroxytryptamine, an oxidative product of 5-HT. These metabolites can later result in the oxidation of proteins, lipids and sugars in DA and 5-HT rich neurons (Cruickshank & Dyer, 2009) and contribute to neural cell damage in the brain (Brown *et al*., 2003). ROS associated with MA is also thought to activate the Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, which can result in neuronal apoptosis (Vearrier

et al., 2012). Reactive species can also be the result of other processes, including GLU release, mitochondrial dysfunction and NO production, all shown in Figure 2-7 (Riddle *et al*., 2006). The fact that MA administration may result in oxidative stress as well as inflammation bears a striking similarity to schizophrenia (see § 2.2). This again emphasizes that by compounding on an already compromised system in susceptible individuals, MA may precipitate a psychotic event or even the development of schizophrenia.

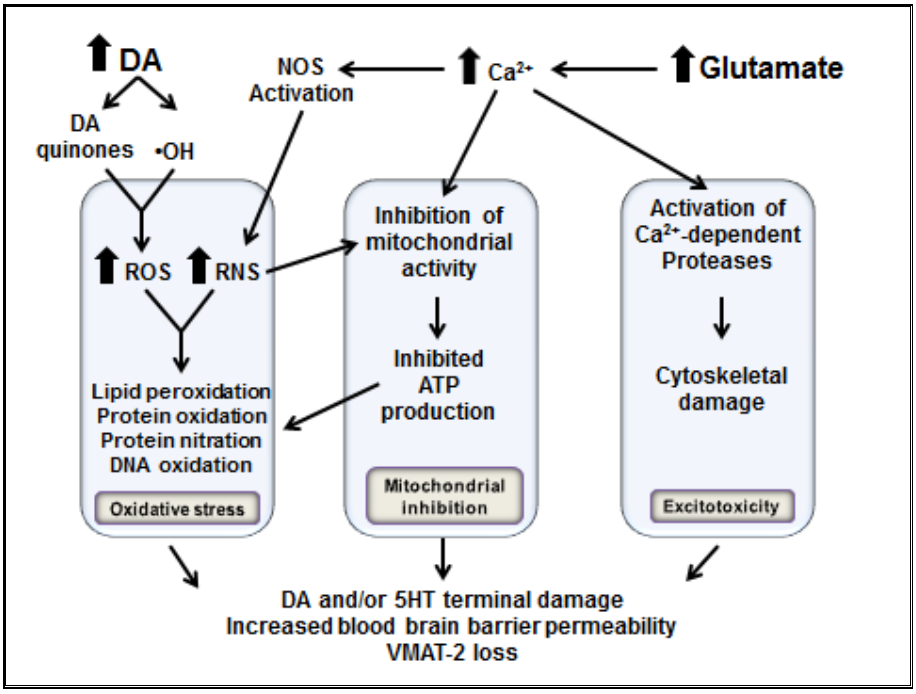

Figure 2-7: **Neurobiological causes and consequences of MA toxicity. MA administration results in an increase in DA where the excess DA is oxidized, producing DA quinones. ROS and RNS are increased, contributing to oxidative stress. MA induced increases in GLU also contributes to the neurodegenerative effects of MA. MA – methamphetamine; ROS – Reactive oxygen species; RNS – Reactive nitrogen species; DA – Dopamine. (Adapted from Riddle *et al*., 2006)**

2.3.5.2 MA and mitochondrial dysfunction

MA abuse is likely to result in mitochondrial dysfunction, which is thought to be an important consequence of dopaminergic depletion (see Fig. 2-8; Thrash *et al*., 2009). Mitochondria produce cellular energy in the form of adenosine triphosphate (ATP) that is used in the cell for a variety of functions (Riddle *et al*., 2006). However, when ATP production is inhibited it can result in injury to cells, including apoptosis or necrosis (Thrash *et al*., 2009). The production of ATP is regulated by enzymatic reactions like the citric acid cycle and electron transport chain (ETC; Riddle *et al*., 2006). Many studies have recorded MA-induced inhibition of the mitochondrial ETC enzyme complexes, including complex I, complex II-III and complex IV (Yamamoto *et al*., 2010). The exact mechanism of the impairment of mitochondrial function is not yet known, but ROS and RNS are thought to be involved. GLU-induced increase in intracellular Ca^{2+} will also

contribute to mitochondrial dysfunction (Yamamoto *et al*., 2010; Brown & Yamamoto, 2003). The subsequent formation of ROS by MA can cause mutations in mitochondrial DNA resulting in epigenetic effects, dysfunction in the respiratory chain and cell damage via apoptosis. Since MA is lipid soluble, it can diffuse into mitochondria and be preserved for long periods of time in the cytoplasm of the mitochondria, thereby disrupting the chemical gradient necessary for the correct functioning of the ETC (Thrash *et al*., 2009).

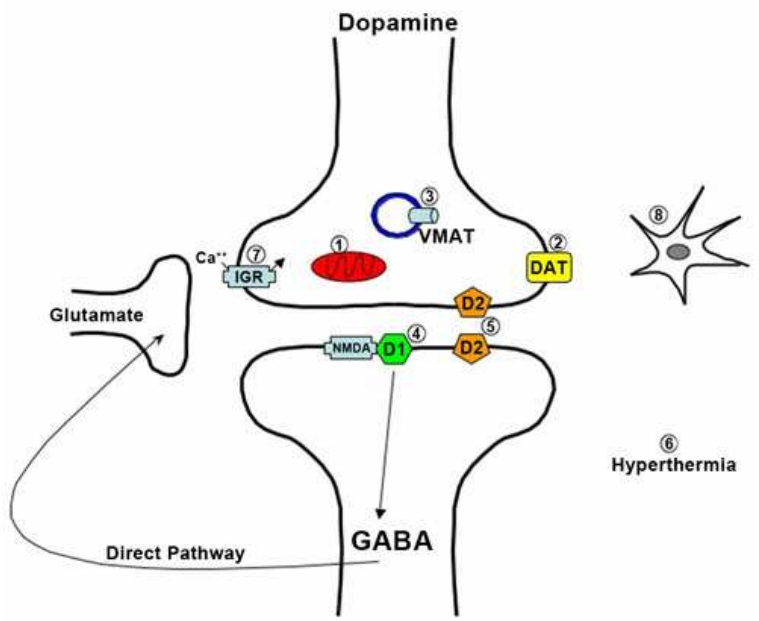

Figure 2-8: **Schematic of the possible components involved in MA induced neurotoxicity. (1) mitochondrial dysfunction; (2) decrease in DAT (dopamine transporter) activity; (3) alterations in VMAT-2 (vesicular monoamine transporter-2) activity; (4) D_1 receptor involvement; (5) D_2 receptor involvement; (6) hyperthermia; (7) ionotropic GLU receptor (IGR) involvement in mitochondrial damage and (8) microglial activation and ROS formation (Riddle *et al*., 2006).**

2.3.5.3 MA and hyperthermia

High doses of MA cause hyperthermia, and is strongly linked to the occurrence of neurotoxicity and mortality (Fig. 2-8; Yamamoto *et al*., 2010; Brown *et al*., 2003). Furthermore, hyperthermia can enhance the effects of MA, whether they are mediated by enzymatic or nonenzymatic processes (Yamamoto *et al*., 2010). Hyperthermia potentiates decreases in DA and TH and can also interact with other systems and processes, such as oxidative stress and astrocytosis, resulting in an increase in catecholaminergic and glutamatergic neurotransmission (Brown *et al*., 2003), and eventually neurotoxicity (Yamamoto *et al*., 2010). Hyperthermia increases the formation of free radicals and DA quinones and has also been linked to the dysregulation of DAT function. This is supported by evidence that the increase in hydroxyl radicals caused by MA administration can be blocked when hyperthermia is prevented. Thus, it has been suggested that hyperthermia contributes to the MA-induced formation of ROS (Krasnova &

Cadet, 2009). Hyperthermia following MA administration is seen more prominently in the brain, suggesting that neuronal activation due to MA may contribute to the rise in temperature (Brown *et al*., 2003; Maxwell, 2005). A study by Brown *et al* (2003) suggested that MA-induced social or arousing situations may exacerbate hyperthermia, increasing the risk of adverse effects.

2.3.5.4 MA and the glutamatergic system

The glutamatergic system has been implicated in the pathophysiology of schizophrenia, as well as of MA-induced neurotoxicity (see § 2.2 and Fig. 2-7 & Fig. 2-8; Frost & Cadet, 2000). Moreover, MA has been shown to increase extracellular concentrations of GLU in the hippocampus and striatum (Tata & Yamamoto, 2007). GLU is a major excitatory neurotransmitter in the CNS and is important in the modulation of excitatory neurotransmission (Tata & Yamamoto, 2007). Excitotoxicity refers to the processes where cells are damaged or killed as a result of over stimulation of excitatory amino acid receptors (Thrash *et al*., 2009). The process of excitotoxicity includes many events where intracellular Ca^{2+} may be increased, resulting in Ca^{2+}-dependent enzyme activation (see Fig. 2-7), GLU release and subsequent activation of GLU NMDA receptors (Yamamoto *et al*., 2010). GLU binds two types of receptors, namely metabotropic and ionotropic receptors. Excitatory effects are mediated by the ionotropic receptors, which include NMDA, α-amino-5-hydroxy-3-methyl-4-isoxazde propionic acid (AMPA) and kainic acid (KA) receptors (Wong & Van Tol, 2003). DA cells contain NMDA and AMPA receptors that are activated by GLU. The subsequent increase in Ca^{2+} may damage mitochondria, culminating in neurotoxicity and neurodegeneration (Fig. 2-7). GLU-mediated activation of NMDA receptors also activates NO synthase (NOS) to produce NO, a key neuromodulator and second messenger. Under conditions of oxidative stress NO reacts with superoxide to form the highly reactive RNS peroxynitrite (see Fig. 2-7; Riddle *et al*., 2006; Yamamoto *et al*., 2010; Frost & Cadet, 2000; Thrash *et al*., 2009). Moreover, NO has been linked to the nitration of proteins associated with DA and 5-HT terminals, resulting in excitotoxicity. Altered NO signaling is also evident in schizophrenia (Bitanihirwe & Woo, 2011) and has been demonstrated in the rat striatum after amphetamine injection (Wang & Lau, 2001). Furthermore, activation of apoptotic pathways, mitochondrial and endoplasmic reticulum (ER) dysfunction and damage to DNA are also known excitotoxic events mediated by NO (Yamamoto *et al*., 2010). These data therefore suggest that NO, which is necessary for normal neurological function and transmission, can become neurotoxic under conditions associated with MA abuse.

2.3.5.5 MA and inflammation

Inflammation in the brain can promote neurotoxicity in areas rich in DA and 5-HT terminals, while MA administration is known to promote the release of inflammatory mediators, probably via a GLU receptor-mediated response (Yamamoto *et al*., 2010), resulting in the activation of

microglia (Yamamoto & Raudensky, 2008). Activated microglia increases the expression of inflammatory mediators, including pro-inflammatory cytokines interleukin 1β, interleukin 6 and tumor necrosis factor α (TNF-α; Yamamoto *et al*., 2010; Yamamoto & Raudensky, 2008) and monocyte chemoattractant protein (Kuczenski *et al*., 2007). Microglial activation plays an important part in MA-induced inflammation, especially since neurotoxic amphetamines are known to initiate activation of microglia, whereas the latter also usually precedes the appearance of neuronal DA damage in the striatum (Yamamoto & Raudensky, 2008). It is interesting then that schizophrenia too is associated with altered immune function and evidence of a pro-inflammatory state (Leonard *et al*., 2012).

2.3.5.6 Blood-brain barrier disruptions and HIV

Recent studies have demonstrated that MA exposure can increase the permeability of the BBB. This happens in several regions, including the striatum, cortex and cerebellum, and can contribute to degeneration of neurons by allowing easier permeation of toxic substances into the brain, such as that following hyperthermia and oxidative stress. These in turn can further lead to the breakdown of the BBB, making the brain more vulnerable to infections, like HIV. Indeed, HIV and MA both cause damage to tight junction proteins that make up the BBB (Yamamoto *et al*., 2010), whereas clinical studies have confirmed the positive correlation between HIV and MA abuse (Ferris *et al*., 2008).

Studies have shown that HIV can cause damage to DA neurons in the nigrostriatum as well as damage to other parts of the brain, such as the prefrontal cortex (Yamamoto *et al*., 2010; Ferris *et al*., 2008). HIV infection, together with MA abuse, also induces deficits in hippocampal and cortical systems. Changes in these brain regions can result in working memory deficits and disrupted memory formation (Kuczenski *et al*., 2007). Areas damaged by HIV are also more vulnerable to MA-induced neurotoxicity, with MA-associated oxidative stress, inflammation and mitochondrial dysfunction possibly contributing to the neuro-damaging effects of HIV. In fact, MA and HIV together can cause a synergistic increase in toxicity to DA terminals in the striatum, as evinced by an increase in oxidative stress markers and inflammation (Yamamoto *et al*., 2010; Ferris *et al*., 2008).

2.3.5.7 MA and environmental stress

Finally, environmental stress may also contribute to the toxic effects of MA (Yamamoto *et al.,* 2010; Matuszewich & Yamamoto, 2004). Studies have found that behavioural responses and responses to chemical challenges, such as MA, may be altered following repeated exposure to environmental stressors. Thus, prior exposure to stress can enhance the locomotor response to MA. MA-induced release and subsequent depletion of DA in the striatum following acute MA administration may also be augmented by pre-exposure to stressors (Matuszewich & Yamamoto, 2004). Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, together with

activation of the immune system, resulting in the release of pro-inflammatory cytokines (Yamamoto *et al*., 2010). In rodents MA has been found to stimulate the release of glucocorticoids resulting in an increase in plasma corticosterone (Herring *et al*., 2008) that can last up to 24 hours (Grace *et al*., 2010a). Consequently, their combined effects may include increased oxidative stress, mitochondrial dysfunction (Yamamoto *et al*., 2010), hyperthermia (Matuszewich & Yamamoto, 2004), inflammation and excitotoxicity (Yamamoto *et al*., 2010) that will further potentiate neurotoxicity and neurodegeneration. For example, people diagnosed with posttraumatic stress disorder have been found to have a high comorbid abuse of substances like MA (Yamamoto *et al*., 2010; Johnson, 2008). In many cases, MA abuse is associated with high levels of chronic stress, augmenting the neurotoxic effects of the drug (Matuszewich & Yamamoto, 2004).

2.3.6 Neurodevelopmental effects of MA

Overwhelming evidence suggests that schizophrenia is a neurodevelopmental disorder, being causally associated with pre-, peri- and post-natal exposure to adverse environmental conditions (see § 2.2). Furthermore, while psychosis first manifests in young adolescents, there is evidence for early life developmental abnormalities in these patients that attests to the presence of an early life stressor or toxin that is antecedent to the development of the disorder (Fone & Porkess, 2008). This is an important consideration when one considers the neurobiology and causes of MA-induced psychosis, especially that MA abuse takes place predominantly during adolescence and is a strong predictor of psychosis later in life (see § 2.3.7) Moreover, dopaminergic systems begin to form as early as the first trimester, so that prenatal exposure to MA could have serious consequences on the development of the brain (McFadden *et al*., 2011) and, as such, lay the foundation for later life bio-behavioural abnormalities that would include an increased susceptibility to psychotic events.

2.3.6.1 Prenatal exposure to MA

The neurotoxic actions of MA have particular relevance in pregnant women. Recent studies have shown an increase of MA abuse among pregnant women and women of childbearing age, thus posing a risk of damage to the developing foetus (Vearrier *et al*., 2012; Arria *et al*., 2006). Dopaminergic and serotonergic neurons can be detected in the human foetus from as early as 5 weeks of gestation (Frost & Cadet, 2000). Importantly, many women of childbearing age and who are substance abusers list amphetamines as their primary drug of abuse (Piper *et al*., 2011; Grace *et al*., 2010a). MA crosses the placenta and it is therefore inevitable that the foetus will be exposed to the drug (Grace *et al*., 2010a). Indeed, when compared to healthy children, children exposed to MA prenatally have distinct neurochemical and structural brain differences, especially in the striatum (Chang *et al*., 2007; Vearrier *et al*., 2012). These differences can result in poor performance in attention and memory tests described in these children (Vearrier

et al., 2012). Williams *et al* (2004) have stated that the abuse of even low doses of MA during pregnancy can later alter the child's ability to learn.

In a study where MRI spectroscopy was performed on children exposed to MA *in utero*, the investigators found increased concentration of creatine and GLU/glutamine in the striatum, suggesting an increase in cellular metabolic activity that may persist for years after exposure (Williams *et al*., 2004).

Other problems associated with prenatal MA abuse include an increased risk of having CNS birth deficits, increased premature delivery and neonatal mortality, while neonates have been found to present with ventricular enlargement, haemorrhages, elevated white-matter density (Piper *et al*., 2011) and possible amniotic fluid embolisms (Vearrier *et al*., 2012). The size of the putamen, caudate (McFadden *et al*., 2011), globus pallidus and hippocampus are also smaller (Grace *et al*., 2010a; Chang *et al*., 2007) in MA-exposed infants. Studies also show maternal anaemia, pre-eclampsia and premature membrane rupture associated with the abuse of MA during pregnancy. Other birth deficits may include cardiac defects, hydrocephalus (Cruickshank & Dyer, 2009; Phupong & Darojn, 2007) and a higher risk of developing intraventricular hemorrhage (Vearrier *et al*., 2012).

It is also important to consider other factors that may contribute to the development of abnormal behaviours in children exposed to MA *in utero* (Piper *et al*., 2011), such as lower rates of prenatal care in pregnant women abusing the drug (Cruickshank & Dyer, 2009), while in most cases the postnatal environment of the child is sub-optimal. Behavioural outcomes that may develop due to MA exposure include increased stress, a decrease in arousal and a decrease in the quality of movement (Vearrier *et al*., 2012). It is more likely for women abusing drugs to have higher rates of psychiatric disorders, as well as a history of sexual or emotional abuse and in many cases domestic violence (Piper *et al*., 2011; Maxwell, 2005).

Children exposed to MA prenatally are associated with a greater incidence of cognitive impairments, such as difficulties with attention and visual motor integration (McFadden *et al*., 2011; Grace *et al*., 2010a). Studies show that people who have been exposed to prenatal MA have deficits in verbal and/or spatial memory (Grace *et al*., 2010a), as well as in motor performance, confirming the presence of long-term cognitive deficits (O'Dell *et al*., 2011). Deficits in learning, episodic memory and information processing speed have also been documented (Cruickshank & Dyer, 2009). Neurobehavioural deficits may persist well into adulthood (Cruickshank & Dyer, 2009), so that prenatal exposure to MA is associated with a greater risk of developing behavioural abnormalities later in life, including failure to adjust in a social environment, exhibiting aggressive behaviour and poor performance on psychometric tests (Frost & Cadet, 2000).

2.3.6.2 Postnatal exposure to MA

The incidence of MA abuse is the highest amongst adolescents between the ages of 14 and 18 years (Kokoshka *et al*., 2000). Since chronic adolescent MA abuse has a significant causal association with psychotic symptoms or MA psychosis later in life (Vos *et al*., 2010), this emphasizes the contributory role of postnatal MA exposure during adolescence to the development of late life psychopathology (see § 2.3.7.).

A particularly troubling aspect of chronic MA abuse is its association with neurotoxicity (Grace *et al*., 2010b) and neurocognitive impairment that can persist even in the absence of continued MA use (Vearrier *et al*., 2012). In this regard, there is evidence that dendritic structures in the brain may be modified, especially in the prefrontal cortex and the nucleus accumbens (Fig. 2-6; Frost & Cadet, 2000). Impairment of attention may be a result of neurochemical changes in frontostriatal brain regions. In the corpus callosum there are also microstructural changes in the white matter (Vearrier *et al*., 2012; Arria *et al*., 2006).

When considering the impact of MA abuse across the life-span, it is troubling to note that MA neurotoxicity has been linked to an increased risk of developing neurodegenerative disorders like Parkinson's disease (PD) (Cruickshank & Dyer, 2009; Garwood *et al*., 2006), Alzheimer's disease, amyotrophic lateral sclerosis (ALS) (Grant *et al*., 2011; Jayanthi *et al*., 2004) and neurocognitive disorders associated with HIV (Grant *et al*., 2011). People abusing MA on a regular basis have a higher risk of developing PD later in life, as much as 8 times higher than in the normal population (Cruickshank & Dyer, 2009).

2.3.7 MA abuse and psychiatric illness

Up till now it has become evident that MA abuse may have significant causal association with a number of neurological and psychiatric illnesses, especially schizophrenia. Since the connection between MA abuse and schizophrenia is the focus of the current study, it warrants a more detailed discussion.

Many risk factors can contribute to the development of schizophrenia, and drug abuse and social isolation are but two important environmental (non-inheritable) factors contributing towards its development (Seeman, 2011). Studies have shown that chronic abuse or higher doses of MA may lead to the development of psychiatric symptoms referred to as MA psychosis (Nakamura *et al*., 2009; Yui *et al*., 2000; Srisurapanont *et al*., 2011). Indeed, Vos *et al* (2010) declare that the odds of MA users for developing psychotic symptoms or MA psychosis are 11 times higher than the odds of the general population.

MA psychosis is similar to acute paranoid schizophrenia and in most cases indistinguishable (Cruickshank & Dyer, 2009; Yui *et al*., 2000), with deficits in neurocognitive functioning that are

identical (Vearrier *et al*., 2012). In fact, several similarities in both positive and negative symptoms can be seen in MA psychosis and schizophrenia (Srisurapanont *et al*., 2011). Positive symptoms such as hallucinations (auditory or visual), delusions and irrational speech, and negative symptoms including loss of drive, impaired speech, apathy and flattened affect are similar, thus suggesting a shared pathophysiology for schizophrenia and MA psychosis (Srisurapanont *et al*., 2011; Maxwell, 2005). Apart from the positive and negative symptoms observed in schizophrenic patients, deficits in memory and attention can also be observed in these patients (Olsson *et al*., 2012). Many MA abusers also experience delusions of persecution (Grant *et al*., 2011). In both cases, patients experience paranoid hallucinations and their awareness of reality is impaired. Patients suffering from both these disorders are at risk of experiencing a relapse in symptoms, and antipsychotic drugs are effective in the treatment of both disorders (Yui *et al*., 2000).

Volitional disturbances have been documented in MA abusers, including idleness, blunted affect and a loss of spontaneity. Psychosis may or may not disappear after the cessation of MA, but emotional blunting and idleness tend to persist and even increase (Yui *et al*., 2000). When MA is used repeatedly, and particularly if this happens together with experiences that are stressful or threatening, abusers may be more prone to a relapse of paranoid hallucinations, and even mild stressors may precipitate such events (Yui *et al*., 2000).

MA-induced psychosis can be divided into 2 categories, viz. the transient and the persistent type. The transient type of psychosis is characterized by acute recurrences of psychosis and is mostly due to the abuse of MA or stressors (Nakamura *et al*., 2009). Stressors include severe insomnia, incarceration or even the consumption of large amounts of alcohol (Cruickshank & Dyer, 2009; Grant *et al*., 2011). The increase in NA due to stress and also an increase in DA neurotransmission have been suggested to contribute to the recurrence of psychotic symptoms (Grant *et al*., 2011). If, however, a person abstains from MA abuse, the symptoms can disappear within a month. In the case of the persistent type, symptoms can last for more than a month after MA abuse has been stopped. This is an indication of permanent damage to the brain, resulting in a schizophrenia-like psychotic state, even without the concomitant use of MA (Nakamura *et al*., 2009). These individuals exhibiting persistent psychosis are 3 times more likely to also have a familial history of schizophrenia (Cruickshank & Dyer, 2009) or a schizotypal personality preceding the development of the disease (Grant *et al*., 2011). Other risk factors for developing persistent psychosis include a young age when the person started abusing the drug, with more severe abuse (Vearrier *et al*., 2012; Grant *et al*., 2011), with longer duration of abuse and dependence, with the intravenous route of administration (Darke *et al*., 2008; Grant *et al*., 2011) with a history of other neurological disorders (such as attention-deficit/hyperactivity disorder (ADHD), a learning disorder (Vearrier *et al*., 2012; Grant *et al*., 2011), or if the person suffered a head injury or was born prematurely (Grant *et al*., 2011). MA

abusers with psychosis tend to be more prone to develop major depression, anti-social personality disorder and alcoholism (Chen *et al*., 2003; Grant *et al*., 2011).

Psychosis is often the result of the brain being sensitized to the psychotogenic action of the drug following chronic abuse (Nakamura *et al*., 2009). This behavioural sensitization can result in a relapse of psychosis, even with small doses of MA (Vearrier *et al*., 2012). When a person is repeatedly exposed to psychostimulants such as MA, the abuse can result in cellular neuroadaptation in the brain. This effect can be long-term, leading to the emergence of certain behaviours that can be associated with addiction. Animal studies support clinical observations that repeated administration of MA or amphetamine can result in behavioural sensitization (Suzuki *et al*., 2004; Lin *et al*., 2002). This includes progressive increases in behaviour-like locomotion, rotational behaviour and stereotypy (Suzuki *et al*., 2004). The development of psychosis is usually accompanied by social withdrawal (Darke *et al*., 2008). Behavioural sensitization can remain for several months and can be used as a model of addictive behaviour in humans, like a craving for drugs (Suzuki *et al*., 2004), or as a model for paranoid schizophrenia and psychostimulant psychosis (Lin *et al*., 2002). The exact mechanism of behavioural sensitization is not clear, although some studies claim sensitization is the result of hyperactivity in the mesoaccumbens and also the mesostriatal dopaminergic system (Lin *et al*., 2002). Neurotransmitters such as substance P and sigma receptors have been implicated in sensitization. Chronic MA abuse results in a decrease in substance P receptor binding, whereas the administration of sigma receptor antagonists protects against the development of sensitization. DA supersensitivity is also progressively enhanced with continuous administration of MA, contributing to MA psychosis (Grant *et al*., 2011).

MA-induced psychotic symptoms and memory deficits generally correlate with neurotoxicity in the striatum. Reduced DAT density in the frontal cortex (Cruickshank & Dyer, 2009), nucleus accumbens and striatum (Grant *et al*., 2011) and globally reduced SERT density is thought to be related to psychosis experienced by abusers (Cruickshank & Dyer, 2009). The reduction in DAT density is also thought to be related to slower motor responses and also a reduced memory performance (Volkow *et al*., 2001a; Grant *et al*., 2011). MA users generally experience psychotic symptoms like paranoia, delusions, auditory hallucinations (Vos *et al*., 2010), odd speech (Cruickshank & Dyer, 2009), pathological hostility (Vearrier *et al.,* 2012) and sometimes even outbursts of rage (Vos *et al*., 2010). Depressive symptoms and suicidal ideation are also seen in chronic MA abusers. Some people are more at risk of attempting suicide after MA use, including people with a history of MA induced psychosis or depressive disorder or a familial history of psychosis (Vearrier *et al*., 2012).

The severity of the psychiatric symptoms experienced by MA abusers is related to the amount of neuronal damage in the basal ganglia (Vearrier *et al*., 2012; Chang *et al*., 2007). MA abusers

experience changes in brain metabolism and also damage to the brain, resulting in the development of MA psychosis. Because MA psychosis and schizophrenia are so closely related, studying the neurobiology of MA psychosis may lead to a better understanding of the pathophysiology of schizophrenia (Yui *et al*., 2000). MA-induced psychosis and schizophrenia share a dysfunction of the mesolimbic DA system and prefrontal cortex (Srisurapanont *et al*., 2011), whereas both disorders also share structural changes in the brain, including enlarged basal ganglia and lateral ventricles, and a smaller hippocampal volume (Grant *et al*., 2011). Moreover, as has been described earlier, altered redox status and inflammation are also evident in both disorders.

2.3.8 Modelling schizophrenia and MA psychosis in animals

2.3.8.1 An animal model of MA psychosis?

To develop a better understanding of the neurobiology and also the genetic and molecular basis of psychiatric disorders, animal models have provided a valuable means for acquiring new knowledge and to test novel treatment modalities (Fone & Porkess, 2008). The development of animal models that have translational relevance for our understanding of MA psychosis and its possible treatment strategies is urgently needed. Such an animal model should exhibit the same clinical symptoms as in human MA abusers (face validity), display similar brain changes associated with the use of MA (construct validity) and demonstrate response to antipsychotics as observed in human MA users (predictive validity). Close attention to the behavioural and neurochemical changes in a suitable animal model will lead to a better understanding of the possible mechanisms involved in human MA psychosis. Although a single animal model is often insufficient to provide an accurate representation of all the mechanisms involved in human MA psychosis, it can provide a number of important clues as to its underlying neurobiology and suitable treatment response. Many physiological or environmental precursors can have an effect on the development of psychosis in MA abusers, and these factors should be closely emulated in the model in order to provide robust etiological validity (Grant *et al*., 2011).

When animals are chronically treated with a low dose of MA, they develop behavioural manifestations that closely mimic the human pattern of MA abuse, where psychosis often emerges after high-dose binges of MA. These symptoms may include the development of stereotype behaviour, a reduction in motor activity, a decline in social behaviour and a loss of interest in the surroundings or outside stimuli (Grant *et al*., 2011). Studies have shown that paranoid schizophrenia, MA induced psychosis and the behavioural sensitization induced by MA in rodents share much of the same underlying neuropathology (Ujike, 2002). This response forms the basis for one of the more widely used animal models of schizophrenia, viz. behavioural sensitization induced by psychostimulants such as MA (Ujike, 2002; Grant *et al*., 2011). However, as has been described in previous sections, a widely accepted etiological

theory of schizophrenia is its association with pre-, peri- and postnatal adversity leading to early-life neurodevelopmental abnormalities, with subsequent presentation of schizophrenia later in life. An animal model that has found wide acceptance for the accuracy in mimicking the neurodevelopmental aspects of schizophrenia, as well as for presenting with robust face, construct and predictive validity, is the post-weaning SIR model (Geyer *et al*., 2001; Möller *et al*., 2011).

2.3.8.2 Social isolation rearing

Schizophrenia is a polygenetic and heterogeneous disorder that can be influenced by an adverse environment in early life (Fone & Porkess*,* 2008). Although a single animal model cannot replicate all the symptoms and signs as well as neurobiological and treatment-related characteristics of this disorder, post-weaning SIR of rodents (§ A.2.3) has been found to reproduce many of the behavioural, neurochemical and treatment-related responses akin to schizophrenia. This includes social withdrawal, impaired sensorimotor gating, neophobia and cognitive dysfunction (Fone & Porkess*,* 2008). Furthermore, as a neurodevelopmental model SIR induces a number of structural brain changes in isolated animals that closely resembles that observed in schizophrenia (Fone & Porkess*,* 2008). Recent studies in our laboratory have also found that SIR in rodents is associated with altered frontal cortical NMDA vs. D_1 receptor binding (Toua *et al*., 2010), increased cortico-striatal oxidative stress (Möller *et al*., 2011) and altered kynurenine metabolism. The latter includes reduced plasma levels of the putative neuroprotectant KYNA and elevated levels of the excitotoxin QA (Möller *et al*., 2012). Importantly, these changes can be reversed by antipsychotic treatment (Toua *et al*., 2010; Möller *et al*., 2011; 2012). Interestingly, the latter findings are congruent with that known to occur in MA abuse, e.g. oxidative stress, inflammation and altered DA-GLU signalling. Thus SIR is an ideal model with which to investigate the neurobiology of MA psychosis.

2.4 Synopsis

MA is a fast-growing global problem with significant public health implications. MA is highly addictive and is easily accessible, with the incidence of MA abuse surpassing that of many other widely abused drugs. Next to cannabis, amphetamine-type stimulants are the second most widely abused drug in the world (Vearrier *et al*., 2012). Evidence for neurotoxicity as well as its close similarity with schizophrenia warrants urgent research on the neurodevelopmental effects of MA. In particular, its effects on neurodevelopment and late-life consequences that is relevant. MA abuse also predisposes to the later development of psychosis and schizophrenia, even more so under certain adverse environmental conditions. A limited amount of research data is available on the effects of MA on neurodevelopment, especially pre- vs. postnatal MA exposure and the later development of aberrant behaviour and neurobiological changes.

There are several hypotheses regarding the mechanism by which MA induces neurotoxicity (Kita *et al*., 2003); the most popular being altered DA metabolism and oxidative stress. However, limited work has been done on the exact nature of the redox imbalance induced by MA, and which redox-active pathways are involved in its effects. It is thus necessary for us to understand these mechanisms and to establish whether they may be instrumental in mediating the neurobiological and behavioural features of its abuse. Moreover, it will assist in providing ideas to improve treatment for the adverse effects of MA, and also to promote public awareness of the dangers associated with MA abuse.

The incidence of MA abuse has been shown to be the highest during early adulthood, increasing the likelihood of a profound effect on neuronal development, especially DA and 5-HT neurons. Such early-life changes in neurodevelopment may predispose to later onset neurocognitive changes. In support of this notion, it is evident that prenatal exposure to MA via maternal abuse of the drug contributes to late-life psychopathology.

Mammals that have been exposed to adverse events like social isolation or maternal separation early in life have profound effects in brain development and results in the manifestation of behavioural alterations later in adulthood (Fone & Porkess, 2008). Similar environmental interventions during early life in humans have also been reported to contribute to psychiatric disorders in individuals that are genetically predisposed, including depression and schizophrenia.

The current study examined the effects of chronic MA administration and the possible development of MA induced psychosis later in life. Consequently, the effects of prenatal vs. early postnatal exposure to MA was studied with respect to the later development of behavioural and neurochemical changes that resemble that seen in schizophrenia, viz. deficits in social interaction and sensory motor gating, as well as regional brain monoamine changes and oxidative stress. This aspect of the study focused on normal animals subjected to pre- and postnatal MA exposure. However, in order to determine whether MA-induced psychosis is similar in extent and severity vs. the psychosis typically associated with schizophrenia, the latter group was compared to behaviours induced by SIR, a neurodevelopmental animal model of schizophrenia. Since MA may also sensitize individuals at risk for developing schizophrenia, this study will also investigate whether combining pre- or postnatal MA exposure with animals reared under conditions of social isolation can exacerbate behaviours elicited by SIR. Moreover, because MA abuse is mostly associated with high levels of stress, adding a stressor such as SIR will make the study more authentic. Finally, we also investigated associated effects of the different treatment and rearing conditions on regional brain monoamine metabolites as well as markers of oxidative stress.

Chapter 3 – Article 1

The current dissertation is presented in the "article format", as recognised by the North-West University. This chapter presents the core data as a research article prepared for submission to an appropriate peer-reviewed scientific journal. Accordingly, an article titled:

"Late-life effects of chronic methamphetamine exposure during puberty on behaviour, cortico-striatal monoamines and superoxide dismutase activity in social and isolation reared rats"

has been prepared as a full-length research report for submission to ***Developmental Neuroscience.*** Different from other chapters in this dissertation, the current chapter has been prepared according to the Instructions to the author for this journal (see *Addendum D)*. The references for this chapter are therefore also provided at the end of the manuscript, and not at the end of the dissertation.

The manuscript is presented in the required format prescribed by *Instructions to the Authors,* as outlined on the journal website:

http://content.karger.com/ProdukteDB/produkte.asp?Aktion=JournalGuidelines&ProduktNr=224107&ContentOnly=false, under "Guidelines for authors"

The manuscript will begin with the title, contributing authors and affiliations on a separate page, followed by an Abstract also on a single page. Thereafter will follow the main body of the manuscript, including: Introduction, Materials and methods, Results, Discussion and conclusion, Acknowledgements, References, Legends to Tables, Tables, Legends to Figures and Figures. However, for the benefit of the reader, all figures and tables have been included in the text and not at the end of the manuscript as normally required by the Journal.

L. Strauss undertook all the behavioural, neurochemical and statistical analyses, and wrote the first draught of the manuscript. B.H. Harvey designed and supervised the study and assisted in the interpretation of the study data, as well as finalized the manuscript for publication. D.J. Stein and C.B. Brink advised on the study design and proof read the final manuscript.

Title of article

Late-life effects of chronic methamphetamine exposure during puberty on behaviour, cortico-striatal monoamines and superoxide dismutase activity in social and isolation reared rats

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Abstract

Methamphetamine (MA) is the second most widely abused drug world-wide after cannabis. Much of this phenomenon is due to its low manufacturing cost and accessibility. Chronic MA abuse results in oxidative stress and a psychosis indistinguishable from paranoid schizophrenia. Social isolation rearing (SIR) is a valid neuro-developmental animal model of schizophrenia. We investigated the ability of chronic MA administration to modify schizophrenia-like behavioural and neurochemical changes induced by SIR. New-born pups from MA-naive female Wistar rats were divided into SIR and group-reared animals immediately post-weaning (on postnatal day +21). Young rats subsequently received either saline (2 ml/kg s.c. bid) or an escalating dose of MA (0.2 mg/kg s.c. bid – 6 mg/kg s.c. bid) for 16 days from postnatal day +35 until postnatal day +50. On postnatal day +78, male rats were tested for deficits in social interactive behaviours and prepulse inhibition (PPI), with brains dissected after sacrifice for analysis of regional brain monoamines and superoxide dismutase (SOD) activity. SIR significantly reduced outward-directed social behaviours (rearing time, staying together, approaching, anogenital sniffing), increased self-directed behaviour (self-grooming, locomotor activity) and induced profound deficits in PPI. Similarly, pubertal MA exposure in group-reared and SIR animals induced similar deficits in PPI and outward- and self-directed social behaviours, although MA did not exacerbated the response to SIR. SIR and MA + group-rearing significantly increased frontal cortical DA levels. DA in the MA + SIR combination was elevated but not significantly so. None of the treatments affected striatal monoamine levels. Animals receiving MA, SIR as well as SIR + MA showed a significant decrease in SOD activity in the frontal cortex, with no additive effects. The data thus demonstrates that MA and SIR separately are similarly effective in evoking schizophrenia-like bio-behavioural changes in rodents, although an additive effect was not evident in SIR animals receiving MA. This paper confirms the psychogenic nature of chronic early life MA exposure.

Keywords

Early postnatal exposure, social isolation rearing, methamphetamine, Wistar rat, prepulse inhibition, social interaction, psychosis, schizophrenia, monoamines, oxidative stress.

3.1 Introduction

The psychostimulant drug methamphetamine (MA) is an analog of amphetamine and is fast becoming a global problem (Kirkpatrick *et al.,* 2012; Yamamoto *et al.,* 2010) with more than 35 million people abusing MA on a regular basis (Vos *et al.,* 2010). MA is cheap and easy to produce from various household chemicals. In Cape Town, South Africa, MA (known locally as "Tik") abuse has drastically increased (Vos *et al.,* 2010). MA is mostly abused by adolescents between the ages of 14 and 18 years (Kokoshka *et al.,* 2000). The drug can be smoked, taken orally or injected (Kirkpatrick *et al.,* 2012) with many users going on MA binges lasting up to 4 days (Cruickshank & Dyer, 2009).

MA acts as a substrate for transporters associated with the uptake of dopamine (DA), serotonin (5-HT) and noradrenalin (NA). MA can diffuse or be taken up by these transporters into nerve terminals, after which it then causes a reverse transport of monoamines resulting in increased synaptic levels of the above monoamines (Yamamoto *et al.,* 2010). MA is a potent releaser of DA, NA and 5-HT (Kirkpatrick *et al.,* 2012; Yamamoto *et al.,* 2010). However, when used chronically MA causes long-term depletion of DA and its metabolites (Imam & Ali, 2001; Grace *et al.,* 2010). MA also inhibits the reuptake of these monoamines, further increasing the synaptic levels of monoamines available for neurotransmission (Yamamoto *et al.,* 2010). It is especially its bolstering effect on dopaminergic transmission that is responsible for its psychotogenic effects.

MA has been shown to increase oxidative stress, especially reactive oxygen- and nitrogen species (ROS & RNS) (Imam & Ali, 2001; Riddle *et al.,* 2006). The increase in oxidative stress as well as inflammation induced by MA administration bears a striking similarity to schizophrenia where both these processes are also closely linked to the pathology of the disorder (Yamamoto *et al.,* 2010; Drzyzga *et al.,* 2006). MA administration results in a decrease of critical antioxidants, including superoxide dismutase (SOD), making neurons more vulnerable to oxidative stress and subsequent damage (Yamamoto *et al.,* 2010; Mirecki *et al.,* 2004; Yamamoto & Raudensky, 2008).

It is not unexpected that chronic abuse of MA can therefore result in psychosis and neurotoxicity (Yamamoto *et al.,* 2010). In fact studies have shown that MA abusers are up to 11 times more likely to develop psychosis than the general population (Vos *et al.,* 2010). MA induced psychosis is similar to acute paranoid schizophrenia and in most cases the two syndromes are indistinguishable (Cruickshank & Dyer, 2009), with symptoms such as paranoia, hallucinations and delusions (Vos *et al.,* 2010). Furthermore, both MA psychosis and schizophrenia share several similarities in positive and negative symptoms, as well as cognitive deficits (Srisurapanont *et al.,* 2011).

Recent studies have indicated that individuals exposed to early life stress or adversity might be more vulnerable to developing late life behavioural abnormalities (Fone & Porkess, 2008). Genetic predisposition, neurotransmitter abnormalities, viral infections, stress, substance abuse, vitamin D deficiency, obstetric complications and altered immune function are implicated in the pathogenesis of schizophrenia (Seeman, 2011). However, schizophrenia is widely regarded as a neurodevelopment disorder, being causally related to pre-, peri- and postnatal adversity (Daenen *et al.,* 2003). As noted earlier, MA abuse often occurs during early adolescence. The question therefore arises whether early life MA abuse places a vulnerable individual at greater risk for developing schizophrenia later in life? Epidemiological studies would suggest that it does (Nakamura *et al.,* 2009; Yui *et al.,* 2000; Srisurapanont *et al.,* 2011; Vos *et al.,* 2010), although this question has never been formerly studied either at clinical or pre-clinical level.

Animal models of early life adversity can help us to better understand the underlying mechanisms that predict the interplay between MA abuse and vulnerability to develop MA psychosis (Dimatelis *et al.,* 2012). It is noteworthy that drug abuse and social isolation are two important non-genetic factors that contribute towards the development of schizophrenia (Seeman, 2011), while post-weaning social isolation rearing (SIR) of rodents is regarded as a valid neurodevelopmental model of schizophrenia (Fone and Porkess, 2008). Indeed, SIR has been found to reproduce many of the behavioural and neurochemical abnormalities observed in schizophrenia, including cognitive dysfunction, social withdrawal, impaired sensorimotor gating and neophobia, as well as demonstrating good predictive validity with respect to response to antipsychotic drugs (Fone & Porkess, 2008). Recently it has also been demonstrated that SIR is associated with cortico-striatal oxidative stress (Möller *et al.,* 2011), therefore resembling the neuropathology of schizophrenia (Bitanihirwe & Woo, 2011) and also MA abuse.

The objective of the current study was to investigate whether chronic MA exposure immediately after weaning evokes similar late-life behavioural, neurochemical and redox-related changes to that induced by SIR, and whether any such bio-behavioural changes are exacerbated in SIR animals receiving MA. Behavioural and neurochemistry markers include effects on sensorimotor gating and social interactive behaviour and effects on cortico-striatal monoamines and SOD activity.

3.2 Materials and methods

3.2.1 Animals

Male Wistar rats, weighing 250 g – 300 g on the day of testing, were used. According to earlier studies validating the SIR model for schizophrenia (see Toua *et al.,* 2010; Möller *et al.,* 2011), at weaning (postnatal day 21; PostND21) animals were randomly separated into group-reared animals (groups of 3-4 rats/cage) and SIR animals (1 rat/cage) and reared under these

conditions for 8 weeks (until PostND77). Rats were reared in identical cages containing sawdust. All animals were housed under conditions of constant temperature (22 ± 1℃) and humidity (50 ± 10%) with a 12:12-h light/dark cycle (lights on 06:00 to 18:00). Food and water were provided ad libitum. During the isolation period, animals were maintained in an environment with no enrichment and minimal handling. Cages were changed once a week with fresh sawdust.

The study and all animal procedures were approved by the Ethics Committee of the North-West University (*approval number: NWU-000105-11-S5*) and were in accordance with the guidelines for the care and use of laboratory animals for research in South Africa.

3.2.2 Drug treatment

Methamphetamine hydrochloride (MA) was dissolved in saline before being administered according to body weight on that specific day. Male rat pups weaned on PostND 21 were divided into groups of SIR rats and group-reared rats. Rats were injected subcutaneously (s.c.) twice a day with either vehicle control (saline) or MA at an escalating dose, starting with 0,2 mg/kg on postnatal day 35 and ending with 6 mg/kg on postnatal day 50 (see Table 3-1; Graham *et al.,* 2008; Keller *et al.,* 2011; Kuczenski *et al.,* 2007). Dosing with MA took place at 9:00 and 15:00 every day for 16 consecutive days. The study consisted of four treatment groups (10 rats/group) designated as follows: Group-reared + saline; Group-reared + MA; SIR + saline; SIR + MA. After behavioural testing from PostND78, all animals were sacrificed by decapitation without any prior use of an anesthetic agent. Thereafter frontal cortices and striata were dissected for use in the neurochemical analyses.

Table 3-1: Escalating dosage regimen for MA from PostND 35 - 50

Day	09:00	15:00
1	0.2 mg/kg	0.4 mg/kg
2	0.6 mg/kg	0.8 mg/kg
3	1.0 mg/kg	1.2 mg/kg
4	1.4 mg/kg	1.6 mg/kg
5	1.8 mg/kg	2.0 mg/kg
6	2.2 mg/kg	2.4 mg/kg
7	2.6 mg/kg	2.8 mg/kg
8	3.0 mg/kg	3.2 mg/kg
9	3.4 mg/kg	3.6 mg/kg
10	3.8 mg/kg	4.0 mg/kg
11	4.2 mg/kg	4.4 mg/kg
12	4.6 mg/kg	4.8 mg/kg
13	5.0 mg/kg	5.2 mg/kg
14	5.4 mg/kg	5.6 mg/kg
15	5.8 mg/kg	6.0 mg/kg
16	6.0 mg/kg	6.0 mg/kg

Dosage regimen for MA treatment over 16 days from PostND 35 to 50

3.2.3 Behavioural tests

Following the vehicle/drug treatments, the animals were housed under their prescribed SIR or grouped housing conditions until PostND78, at which time the behavioural tests were performed, as described below. All behavioural tests were performed sequentially between 1 to 4 hours after the start of the dark cycle (i.e. 19:00 – 22:00).

Prepulse inhibition (PPI) test

Prepulse inhibition (PPI) is used to determine deficits in sensorimotor gating, a well-known abnormality in schizophrenia (Geyer *et al.,* 2001; Möller *et al.,* 2011; Fone & Porkess, 2008). PPI was assessed in two illuminated and ventilated sound–attenuated startle chambers (SR-LAB, San Diego Instruments, San Diego, USA Möller *et al.,* 2011). A stabilimeter system composed of a transparent Plexiglas cylinder (diameter 8.2 cm, length 20 cm) mounted on a Plexiglas frame is present in each startle chamber. The acoustic noise bursts are provided by a speaker mounted 24 cm above the cylinder. A piezoelectric accelerometer mounted below the frame detects and transduces the startle responses of the rat within the cylinder. Stimuli are delivered and startle responses measured during each session using SR-Lab software (San Diego Instruments) running on a Hewlett-Packard personal computer. Startle amplitudes are defined as the average of one hundred 1-ms stabilimeter readings collected from the stimulus onset. The stabilimeter is calibrated before each test.

A protocol using previously described methods (Möller *et al.,* 2011) was used in the current study. The startle session began with a 5-min acclimatization period, with a continuous 68-dB background noise applied throughout the test session, followed by four blocks of trials with either pulse, no-pulse or prepulse trials, with a total of 100 trials (see Möller *et al.,* 2011). % PPI for four prepulse intensities (72, 76, 80 and 84 dB) was used and calculated according to the formula: % PPI = [(Pulse-alone amplitude – Prepulse + pulse amplitude)/Pulse-alone amplitude] x 100 (Ettinger *et al.,* 2005; Cadenhead, 2011). The last 10 trials consisted of a single 40 ms 120 dB pulse-alone startle stimuli. The total of 100 trials was delivered with an average interval of 25 s. The first and last 10 pulse-alone stimuli (BLOCK 1 and BLOCK 4, respectively) and the 20 pulse-alone stimuli that were included in the PPI block itself (BLOCK 2 and BLOCK 3), were used to obtain a measure of mean startle amplitude suggestive of habituation in response to repeated delivery of the startling stimuli (Wang *et al.,* 2003; Van den Buuse *et al.,* 2003; Möller *et al.,* 2011).

Social interaction test

The social interaction test measures deficits in social and self-directed behaviours, a valid behavioural anomaly in schizophrenia (Fone & Porkess, 2008), and was performed as previously described (Möller *et al.,* 2011). In the current study, the SI test was used to assess spontaneous social interactive (outwardly directed) and self (inwardly)-directed behaviours in rodents using the open field test (OFT) (Shimazaki & Chaki, 2005; Ferdman *et al.,* 2007). This test is also performed as a measurement of spontaneous locomotor activity in rodents (Gonzalez *et al.,* 1996; Ferdman *et al.,* 2007). The apparatus used consisted of a black square (70 cm x 70 cm x 40 cm) arena, divided into 35 cm x 35 cm equal squares drawn onto the floor of the arena (Sherif & Oreland, 1994). The arena was illuminated with red light (40 W), with a digital video camera mounted above the arena to record all spontaneous behaviours.

Two rats are placed in the centre of the floor and allowed to adapt for 30s. One of the two rats will be tested for social interaction with an unknown test partner that does not differ by more than 10g in weight. Both members of a test-pair will have received the same treatment as well as the same prior experience. The time spent in active social interactive behaviours, including rearing, approaching and staying with the partner and anogenital sniffing (Gonzalez *et al.,* 1996), as well as self-directed behaviour, including self-grooming and squares crossed (Gonzalez *et al.,* 1996), were scored over a period of 10 min. Subsequent video analyses was performed and scores used for analysis. Testing was performed between 19:00 – 22:00. The evaluator remained outside the testing room during the 10 min testing session. After each test the arena was cleaned with 10 % ethanol solution to remove urine, faeces and odour trails (Ferdman *et al.,* 2007).

3.2.4 Neurochemical analysis

Preparation of brain tissue

To avoid undue effects of behavioural testing on the neurochemical analyses performed, rats were sacrificed by decapitation 24 hours after the final behavioural test and the frontal cortex and striatum immediately dissected out on an ice-cooled glass slab. After removing the olfactory bulb from the cortex and then cutting around the anterior tip of the corpus callosum, the frontal cortical tissue was dissected (Möller, 2009; Toua *et al*., 2009). When dissecting the striatum, the dorsal side of the brain was placed side up. The two cerebral hemispheres were split and the striatum dissected with the corpus callosum as external limits and the external walls of the lateral ventricles as internal limits. The striatum and frontal cortex was then fixed in liquid nitrogen and stored at -80℃ until the day of monoa mine analysis. On the day of assay, the striatum and frontal cortex were removed from the freezer, weighed and allowed to thaw on ice.

Regional brain monoamine analysis

Frozen brain tissue collected after sacrifice was used for quantification of DA, 5-HT and NA levels in the frontal cortex and striatum using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (Harvey *et al.,* 2006; 2010). Monoamine concentrations in the samples were determined by comparing the area under the peak to that of the monoamine standard (isoprenaline; range 5–50 ng/ml; Chemstation Rev. A 06.02 data acquisition and analysis software). Linear standard curves (regression coefficient greater than 0.99) were found in this range. Monoamine concentrations were expressed as ng/mg wet weight of tissue. The limit of quantification (LOQ) for the monoamine assay is 10 ng/ml. The limit of detection (LOD) is 2.5 ng/ml (Harvey *et al.,* 2006).

Analysis of SOD activity

As a key antioxidant enzyme responsible for reducing levels of superoxide in tissue, SOD activity was determined as a measure of oxidative stress. In this assay, an elevation in SOD activity implies an increase in the production of superoxide and is an indicator of tissue redox status. Immediately prior to SOD activity determination, the frozen brain tissue was thawed on ice and weighed. The tissue was then sonicated (2 x 12 seconds, at an amplitude of 14 μ) to prepare a 10 % tissue homogenate with phosphate buffered saline. SOD activity was then determined using a commercially available Superoxide Dismutase (SOD) Activity Assay Kit® (BioVision, Centurion, South Africa). The kit utilizes WST-1 (2-(4-iodophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction by superoxide anion. The rate of this reduction is linearly related to xanthine oxidase (XO) activity, and is inhibited by the presence of SOD in the sample. SOD activity is then quantified by measuring the decrease in colour development by determining the absorbance at 450nm (multiscan plate reader, AEC Amersham), expressed as percentage change.

3.2.5 Statistical analysis

Multiple comparisons relative to control were analysed by two-way analyses of variance (ANOVA) followed by the Tukey post-test. The unpaired Student's t-test (two-tailed) was performed to compare the data from two treatment groups. GraphPad Prism® (version 5.00, San Diego California, U.S.A.) was used for all statistical analyses and graphical representation. A value of $p<0.05$ was taken as statistically significant.

3.3 Results

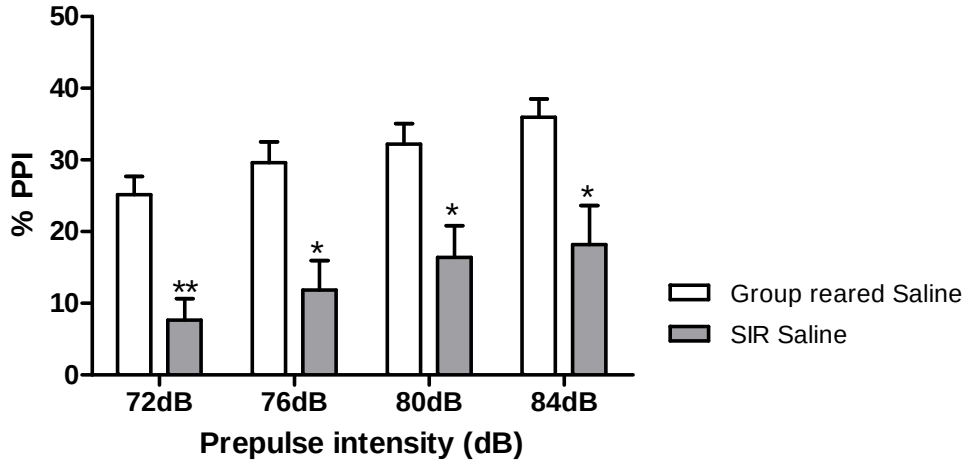
3.3.1 Behavioural studies: Prepulse inhibition (%PPI)

Two-way ANOVA revealed a significant %PPI*housing condition interaction in the saline-treated groups ($F(1,18)=32.899$, $p=0.001$). %PPI was significantly reduced in the SIR rats receiving saline compared to their saline-treated group-reared controls, with a significant overall effect evident at each prepulse intensity: 72dB ($p<0.001$), 76dB ($p<0.003$), 80dB ($p<0.008$), 84dB ($p<0.01$) (Fig. 3-1A).

Significant interactions were evident in the drug treatment study in group-reared and SIR animals: %PPI*treatment ($F(1,36)=10.776$, $p=0.002$), %PPI*housing condition ($F(1,36)=10.187$, $p=0.003$), and treatment*housing condition ($F(1,36)=8.675$, $p=0.006$) (Fig. 3-1B). Tukey post hoc analysis indicated no significant differences in %PPI at each prepulse intensity between group-reared animals receiving MA or SIR animals receiving MA or saline (72 dB, 76 dB, 80 dB, 84 dB; Fig. 3-1B). Overall, a significant decrease in %PPI was noted in group-reared animals receiving MA treatment, as well as SIR animals receiving saline treatment and SIR animals receiving MA, compared to group-reared control animals receiving saline. A significant difference between group-reared saline animals and group-reared animals receiving MA was evident at 72 dB ($p<0.001$), at 76 dB ($p<0.006$) and at 84 dB ($p<0.05$), as well as between SIR animals receiving saline at 72 dB ($p<0.002$), at 76 dB ($p<0.02$) and at 84 dB ($p<0.01$) and between SIR animals receiving MA treatment at 72 dB ($p<0.001$), at 76 dB ($p<0.003$) and at 84 dB ($p<0.02$) (Fig. 3-1B). Group-reared MA, SIR+saline and SIR+MA were equally effective at reducing %PPI vs. group-reared controls (+saline), although were not different to one another.

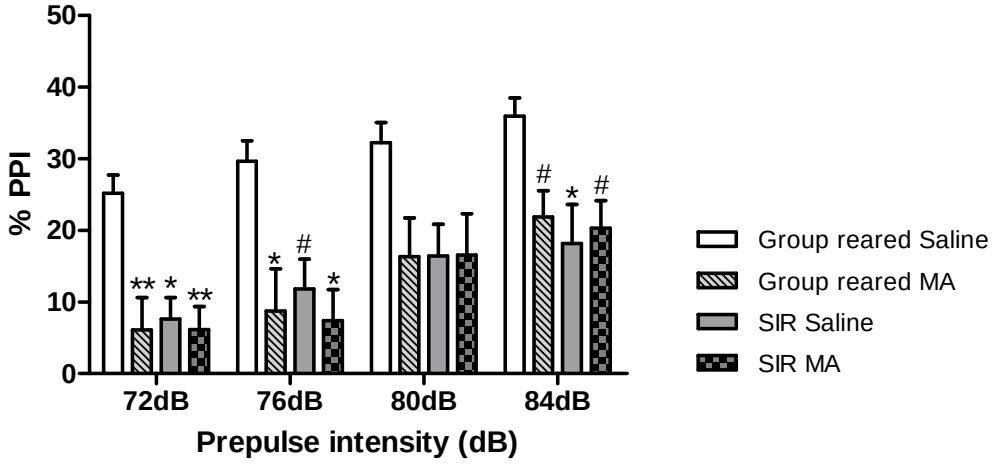
Figure 3-1

A



Statistics: **p<0.001 vs. group-reared (Student's t-test (two-tailed))
***p<0.01 vs. group-reared (Student's t-test (two-tailed))**
see text for precise p values

B



Sensorimotor gating at prepulse intensities as indicated, in (A) the non-treatment cohort (n=10/group) and (B) in group-reared and SIR rats in the drug treatment cohort (n=10/group), as determined by for percentage prepulse inhibition (%PPI). The unpaired Student's t-test (two-tailed) was performed to compare the data of two treatment groups in the non-treatment cohort. In the treatment cohort a two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of p<0.05 was taken as statistically significant. Statistics: **p<0.001 vs. group-reared saline (Two-way ANOVA, Tukey), *p<0.01 vs. group-reared saline (Two-way ANOVA, Tukey), #p<0.05 vs. group-reared saline (Two-way ANOVA, Tukey), at 72 dB, 76 dB, 80 dB and 86 dB respectively

3.3.2 Behavioural studies: Social interaction studies

In the saline-treated cohort, SIR induced a significant attenuation of social- and self-directed behaviours vs. grouped housed controls (data not shown). These two groups did not form a separate study and as such were incorporated into the drug-treated cohort for analysis. In the drug treatment cohort, two-way ANOVA revealed significant cross-group interactions with respect to time spent rearing ($F(1,36)=4.848$, $p=0.034$), approaching ($F(1,36)=3.278$, $p=0.006$), staying together ($F(1,36)=18.454$, $p<0.0001$) and anogenital sniffing ($F(1,36)=11.321$, $p=0.002$) (Table 3-2).

Rearing: Post hoc analysis with the Tukey test revealed a significant decrease in rearing for the group-reared animals receiving MA ($p<0.006$), the SIR animals receiving saline ($p<0.002$) and the SIR animals receiving MA ($p<0.001$) compared to the group-reared control animals receiving saline. However, group-reared MA, SIR + saline and SIR + MA groups were equally effective in this regard (Table 3-2).

Approaching: A significant decrease in approaching in group-reared animals receiving MA ($p<0.007$), SIR animals receiving saline ($p<0.005$) and SIR animals receiving MA ($p<0.001$) was evident compared to group-reared control animals receiving saline (Table 3-2). However, group-reared MA, SIR + saline and SIR + MA groups were equally effective in this regard (Table 3-2).

Staying together: A significant decrease in staying together in group-reared animals receiving MA ($p<0.001$), SIR animals receiving saline ($p<0.001$) and SIR animals receiving MA ($p<0.001$) was evident compared to group-reared control animals receiving saline (Table 3-2). However, group-reared MA, SIR + saline and SIR + MA groups were equally effective in this regard (Table 3-2).

Anogenital sniffing: A significant decrease in anogenital sniffing in group-reared animals receiving MA ($p<0.001$), SIR animals receiving saline ($p<0.001$) and SIR animals receiving MA ($p<0.001$) was evident compared to group-reared control animals receiving saline (Table 3-2). However, group-reared MA, SIR + saline and SIR + MA groups were equally effective in this regard (Table 3-2).

When considering self-directed behaviours, two-way ANOVA revealed significant cross-group interactions for self-grooming ($F(1,36)=2.709$, $p=0.018$) and squares crossed ($F(1,36)=1.133$, $p=0.03$) (Table 3-2).

Self-grooming: Post hoc analysis with the Tukey test revealed a significant increase in self-grooming in group-reared animals receiving MA ($p<0.003$), in SIR animals receiving saline ($p<0.004$) and in SIR animals receiving MA ($p<0.001$) compared to the group-reared control animals receiving saline (Table 3-2). However, group-reared MA, SIR + saline and SIR + MA groups were equally effective in this regard (Table 3-2).

Squares crossed: A significant increase in squares crossed in SIR animals receiving saline ($p<0.003$) and in SIR animals receiving MA ($p<0.001$) was evident compared to the group-reared control animals receiving saline (Table 3-2). Although group-reared MA treated animals showed an elevation in this parameter, this did not differ significantly from saline treated group-reared controls (Table 3-2). Saline or MA treated SIR groups were equally effective in increasing this parameter vs. group-reared controls receiving saline (Table 3-2).

Table 3-2: Behavioural analysis in group-reared and SIR animals receiving either saline or MA

Parameter measured	GR Saline Mean ± SEM	GR MA Mean ± SEM	SIR Saline Mean ± SEM	SIR MA Mean ± SEM
Behaviour:				
Social interaction				
Rearing (s)	33.7 ± 1.693	23.7 ± 2.477 *	22.3 ± 1.726 *	21.7 ± 2 .499 **
Approaching (s)	10.6 ± 0.945	6.9 ± 0.623 *	6.8 ± 0.917 *	6 ± 0.667 **
Staying together (s)	41.2 ± 4.839	10.4 ± 0.476 **	13.9 ± 1.748 **	5.4 ± 0.499 **
Anogenital sniffing (s)	27.4 ± 2.963	12.1 ± 0.875 **	13.5 ± 1.688 **	10.3 ± 0.731 **
Self-interaction				
Self-grooming (s)	4.2 ± 0.593	7.4 ± 0.521 *	7.3 ± 0.844 *	8.4 ± 0.542 **
Squares crossed	90.7 ± 10.397	120.2 ± 11.280	142.6 ± 11.728 *	150.3 ± 6.833 **

Social interactive and self-directed behaviours in group-reared and SIR rats in the treatment cohort (n=10/group): time spent rearing, time spent approaching, time spent together, time spent anogenital sniffing, time spent self-grooming and squares crossed (locomotor activity). A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. Two-way ANOVA followed by Tukey was performed to compare the different drug treatment groups with one another. All data is presented as averages ± S.E.M. with a value of $p < 0.05$ taken as statistically significant Statistics: **p<0.001 vs. group-reared saline (Two-way ANOVA, Tukey), *p<0.01 vs. group-reared saline (Two-way ANOVA, Tukey), GR – Group-reared; SIR – Social isolation reared; MA – methamphetamine

3.3.3 Neurochemical studies

Regional brain monoamines

As noted above, SIR and group-reared cohorts receiving saline (data not shown) were incorporated into the drug-treated cohort for analysis. Two-way ANOVA revealed a significant cross-group interaction in the frontal cortex of the drug treatment cohort with respect to DA ($F(1,34)=8.890$, $p=0.005$), but not with 5-HT or NA (Table 3-3). No significant interactions were noted in the striatum (Table 3-3). Indeed, post hoc analysis with Tukey revealed no significant differences regarding NA and 5-HT in either the frontal cortex or striatum (Table 3-3). However, a significant increase in DA was observed in the frontal cortex of group-reared animals receiving MA ($p<0.001$) as well as in SIR animals receiving saline ($p<0.05$) (Table 3-3) when compared to

their group-reared controls. DA levels were noticeably elevated in this brain region in SIR animals receiving MA although narrowly missed statistical significance (Table 3-3).

Table 3-3: Regional brain monoamines in group-reared and SIR animals receiving either saline or MA

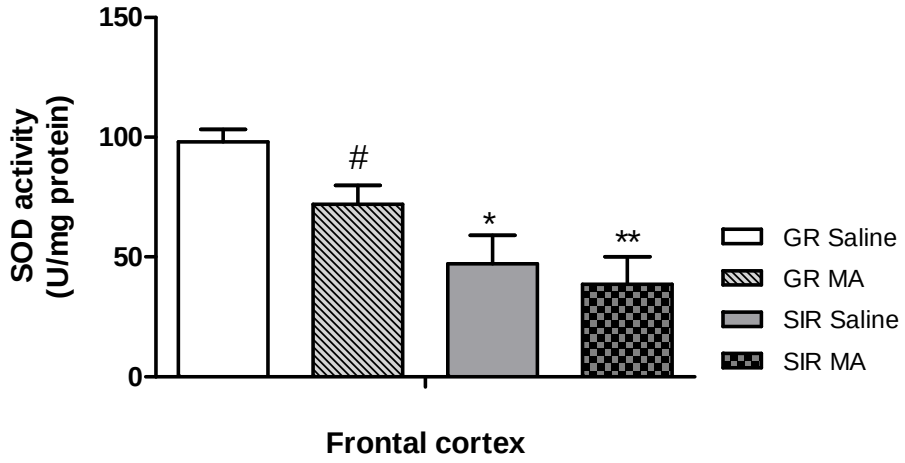
Parameter measured	GR Saline Mean ± SEM	GR MA Mean ± SEM	SIR Saline Mean ± SEM	SIR MA Mean ± SEM
Monoamines				
Frontal cortex				
NA	209.837 ± 9.65	201.880 ± 7.37	214.737 ± 10.08	198.130 ± 5.64
DA	89.25 ± 42.56	541.882 ± 47.15 **	308.836 ± 90.15 #	272.347 ± 117.92
5-HT	193.19 ± 11.65	186.612 ± 12.26	270.721 ± 84.96	269.547 ± 94.55
Striatum				
NA	166.7 ± 6.27	171.959 ± 12.64	162.565 ± 11.9	160.733 ± 12.8
DA	3050.37 ± 208.96	3101.490 ± 168.18	2828.295 ± 317.99	3552.013 ± 259.17
5-HT	273.52 ± 22.4	209.488 ± 12.27	272.844 ± 24.97	306.854 ± 16.89

Frontal cortex and striatum monoamines in group-reared and SIR rats in the treatment cohort (n=10/group): NA, DA and 5-HT. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. Two-way ANOVA followed by Tukey was performed to compare the different drug treatment groups with one another. All data is presented as averages ± S.E.M. with a value of p<0.05 taken as statistically significant. Statistics: **p<0.001 vs. group-reared saline (Two-way ANOVA, Tukey), #p<0.05 vs. group-reared saline (Two-way ANOVA, Tukey), GR – Group-reared; SIR – Social isolation reared;
MA – methamphetamine

Superoxide dismutase activity

Due to a shortage of brain tissue, SOD activity was only determined in frontal cortex where the most noticeable monoaminergic changes were evident. As noted above, SIR and group-reared cohorts receiving saline (data not shown) were incorporated into the drug-treated cohort for analysis. In the drug treatment cohort (Fig. 3-2), two-way ANOVA analysis revealed the following significant interactions: treatment*brain region ($F(1,27)=3.405$, $p=0.05$) and treatment*housing condition ($F(1,27)=20.297$, $p<0.001$). Post hoc analysis with Tukey showed a significant reduction in SOD activity in the frontal cortex of group-reared animals receiving MA ($p=0.05$) as well as in SIR animals receiving saline ($p=0.002$) or MA ($p<0.001$) (Fig. 3-2). Group-reared MA treatment appears less robust in decreasing SOD activity vs. group-reared controls receiving saline than SIR + saline or SIR + MA, although statistically they were not different from one another.

Figure 3-2



Concentration of SOD activity in the frontal cortex of socially reared and SIR rats (n=10/group) in the various non-treatment and treatment cohorts. Data are analysed by a two-way ANOVA followed by a Tukey posttest to compare the different drug treatment groups with their respective control. A value of p<0.05 was taken as statistically significant. Statistics: **p<0.001 vs. group-reared saline (Two-way ANOVA, Tukey), *p<0.01 vs. group-reared saline (Two-way ANOVA, Tukey), #p<0.05 vs. group-reared saline (Two-way ANOVA, Tukey)

3.4 Discussion

The most important observations in this study are that chronic MA administration and SIR (no treatment) reduced %PPI and social interactive behaviours (rearing, approaching, staying together and anogenital sniffing) and increased self-directed behaviours (self-grooming and squares crossed). Chronic MA administration as well as SIR had little effect on the cortico-striatal monoamines, with only a significant elevation of DA in the frontal cortex observed in the group-reared animals receiving MA and the SIR animals receiving saline. Frontal cortical SOD activity on the other hand was significantly reduced in the MA treated animals as well as the SIR animals receiving saline when compared to their group-reared controls.

MA abuse has been linked to a number of neurological and psychiatric illnesses, especially showing noteworthy similarities to acute paranoid schizophrenia (Cruickshank & Dyer, 2009; Yui *et al.,* 2000). Chronic abuse of MA can result in MA-induced psychosis (Nakamura *et al.,* 2009; Yui *et al.,* 2000; Srisurapanont *et al.,* 2011) resulting in positive, negative and cognitive symptoms typical of schizophrenia (Srisurapanont *et al.,* 2011; Maxwell, 2005; Olsson *et al.,* 2012), although the mechanisms involved in this association remain to be established. Animal models can provided a valuable means for acquiring new knowledge on the behavioural and neurochemical consequences of chronic MA abuse and how this may increase vulnerability for developing psychosis or schizophrenia later in life (Fone & Porkess, 2008). Consistent with several earlier studies that MA abuse can predispose an individual to the development of

psychosis, this study has demonstrated that chronic MA administration is equally capable of inducing schizophrenia-like behaviours in rats, including profound deficits in %PPI and social interaction, as does SIR, a valid neurodevelopmental animal model of schizophrenia. In agreement with earlier work (Geyer *et al.,* 2001; Möller *et al.,* 2011), SIR alone induced pronounced deficits in social interactive behaviours, such as rearing, approaching, anogenital sniffing and socializing behaviours such as staying together (Table 3-2), as well as increased self-directed behaviours such as self-grooming and locomotor activity (squares crossed; Table 3-2). More notably, however, was that this same range of social interactive behaviours was similarly compromised after chronic MA exposure. Also emphatic is the demonstration that both SIR and MA separately induce severe deficits in %PPI (Fig. 3-1). Since deficits in sensorimotor gating and self-directed and social interactive behaviours closely resemble well-recognized symptoms of schizophrenia (Powell *et al.,* 2009), this confirms that chronic early-life MA abuse indeed is capable of engendering schizophrenia-like behaviour (e.g. psychosis) later in life.

Early-life adverse events like social isolation can affect brain development and behaviour in adulthood, likely contributing to the development of psychiatric disorders such as schizophrenia in genetically predisposed individuals (Fone & Porkess, 2008). Likewise, MA psychosis might also be more likely to develop or be exacerbated in individuals that have experienced psychosocial or environmental stressors (Grant *et al.,* 2011). Thus in the PPI test, SIR animals receiving saline as well as MA showed profound deficits compared to saline-treated group-reared controls (Fig. 3-1A-B), and in some cases (e.g. at 72 dB and 76 dB) these effects were more pronounced (though not significantly) in the SIR group receiving MA than the SIR group receiving saline (Fig. 3-1B). Similarly, with regard to social interaction, more pronounced deficits were observed in the SIR animals receiving MA treatment with regard to rearing, approaching, staying together and anogenital sniffing, although again significance was not attained (Table 3-2). So while MA and SIR separately significantly compromised social interactive behaviours and sensorimotor gating, we were unable to demonstrate more than a numerical advantage in the combined MA+SIR groups.

Because of the close similarity between schizophrenia and MA psychosis, animal studies such as this will provide more insight into the neurobiology of MA toxicity, while at the same time may also lead to a better understanding of the pathophysiology of schizophrenia (Yui *et al.,* 2000). The acute administration of MA results in increased release of monoamines such as DA, 5-HT and NA (Kirkpatrick *et al.,* 2012; Yamamoto *et al.,* 2010), although chronic exposure to the drug often results in a reduction in these central monoamines (Wang *et al.,* 2000). In an attempt to closely simulate the typical binging pattern used by MA addicts, this study has more specifically considered aspects of chronic MA administration and its subsequent effects on regional brain monoamines 28 days after the last MA injection.

While earlier work has demonstrated that acute MA increases regional brain NA (Kirkpatrick *et al.,* 2012), we observed no significant difference in NA levels in either the frontal cortex or the striatum of rats receiving MA (Table 3-3), thus indicating that any effect of MA on brain NA is more likely to be an acute effect. Nevertheless, since our study design did not allow for the determination of NA levels immediately after MA exposure, this remains to be confirmed.

DA was increased in the frontal cortex of group-reared animals receiving MA as well as the SIR animals receiving saline, and also elevated (non-significantly) in SIR+MA animals (Table 3-3). Dopaminergic abnormalities in the frontal cortex have been linked to the cognitive and negative symptoms of schizophrenia (Wong & Van Tol, 2003; Ross *et al.,* 2006). Specifically, studies have indicated that MA-mediated effects on prefrontal cortical DA can modulate cognitive function in MA abusers (Ross *et al.,* 2006; Nordahl *et al.,* 2003), a mechanism that also has transference to the biology of schizophrenia. However, where schizophrenia is more associated with cortical DA hypofunction or hypofrontality (Wong & Van Tol, 2003), here we show that chronic MA induces a nearly 7 fold *increase* in frontal cortical DA. According to our treatment protocol, this response happened 28 days *after* the final MA dose, inferring that this is highly unlikely to be a product of an acute response to MA challenge. In fact, we propose that this response follows a protracted, insidious developmental change in the functioning of cortical DA neurones or alternatively, due to a gradual loss in cortical glutamate-γ-amino butyric acid (GABA)-glutamate function on pyramidal cells disinhibiting down-stream release of DA in the ventral tegmentum (Stahl, 2007). If one considers the effects of SIR alone, it is noteworthy that an earlier study found a significant decrease in frontal cortical D_1 receptor expression in SIR rats, thus in line with D_1 receptor down-regulation following increased DA activity (Toua *et al.,* 2010). This indeed supports an increase in frontal cortical DA described here. Moreover, since SIR and indeed all MA treatment and adverse rearing conditions were a long-term manipulation, supports the argument that an increase in frontal cortical DA levels is a result of long-term adaptive neuroplastic and/or neurodevelopmental changes.

It is interesting that no significant difference in DA was observed in the striatum between the various treatment and rearing groups, including the SIR group (Table 3-3). Since the DA hypothesis of schizophrenia predicts an increase in striatal DA, these findings appear somewhat unusual. However, such a prediction more likely coincides with the precipitation of frank positive symptoms of the illness following an *acute* increase in striatal (nucleus accumbens) DA (Howes & Kapur, 2009). Indeed, although studies have shown increased DA in the striatum after acute MA administration (Leonard, 2003), repeated administration of the drug results in a decrease in striatal DA (Nordahl *et al.,* 2003). Hyperdopaminergic activity in the striatum and nucleus accumbens is proposed to mediate the disruption in PPI as seen in schizophrenia and SIR rats (Featherstone *et al.,* 2007). However, we found no significant differences in striatal DA, possibly indicating a different mechanism involved in the PPI deficits described here. In fact, abnormal

regulation of glutamate is also implicated in the development of PPI deficits (Nakato *et al.,* 2011; Abekawa *et al.,* 2008; Tata & Yamamoto, 2007), as described in more detail below. The lack of significant differences in DA concentration in the striatum between the groups might also reflect a subchronic effect and that more time is needed after the last MA injection to determine if long-lasting effects on DA concentrations would ensue. It is also possible that abstinence from the drug between MA exposure and behavioural testing may result in a reversal of striatal DA changes. In a study by Volkow *et al* (2001), DA transporter (DAT) loss in MA abusers recovered significantly after a period of abstinence, although neuropsychological function did not show marked improvement. This could explain why no differences were observed in some of the MA treated groups (Table 3-3).

Serotonin has long been associated with psychosis, especially considering psychogenic compounds such as lysergic acid diethylamide (LSD). In the case of the latter, its psychosis-inducing actions are purported to be due to its affinity for the 5-HT_2 receptor, were it acts as an agonist, while also acting as an agonist on 5-HT_{1A} receptors (Passie *et al.,* 2008). However, the psychogenic effects of MA have not been directly associated with the serotonergic system, although abnormalities in the 5-HT_{1A} receptor have been implicated in MA psychosis (Kishi *et al.,* 2010). Nevertheless, since 5-HT-DA cross-talk abounds in the frontal cortex as well as in sub-cortical brain regions (see Carlsson *et al.,* 2001), a role for 5-HT in the bio-behavioural responses observed in this study cannot be disregarded. In fact an increase in locomotor activity, often used as a behavioural marker in animal models of schizophrenia, has been associated with increased 5-HT activity (Steed *et al.,* 2011). However, no differences were observed in 5-HT in either the frontal cortex or striatum, although 5-HT was elevated in SIR and SIR+MA animals, albeit not significantly (Table 3-3). While acute administration of MA results in the release of 5-HT (Yamamoto *et al.,* 2010), chronic administration causes long-term depletion of monoamines such as 5-HT and its metabolites (Imam & Ali, 2001; Grace *et al.,* 2010) which indeed may explain our findings. However, it is important to consider that initial effects on 5-HT may explain MA-induced changes in DA described earlier. Frontal cortical 5-HT exerts a powerful modulatory effect on cortical DA activity, with excessive 5-HT inhibiting DA release via 5-HT_{2C} heteroreceptors on DA neurones (Alex & Pehek, 2007). At the same time, however, overt 5-HT activity may also increase DA release via actions on 5-HT_{1A} autoreceptors (Alex & Pehek, 2007). In support of this, increased 5-HT_{1A} receptor density has been found in the prefrontal cortex of schizophrenia patients and several studies indicate that abnormalities in the 5-HT_{1A} receptor are also involved in MA psychosis (Kishi *et al.,* 2010).

Neurotoxicity induced by MA administration, however, typically displays a decrease in 5-HT and DA in the striatum (Fukumura *et al.,* 1998). Tryptophan hydroxylase (TH), the rate-determining enzyme in 5-HT synthesis, is also reduced following MA administration, a response suggested to be mediated by MA-associated oxidative stress leading to decreasing 5-HT synthesis

(Kokoshka *et al.,* 1998). This is an important finding since chronic MA administration can induce apoptosis in the frontal cortex (Nakato *et al.,* 2011; Abekawa *et al.,* 2008). Oxidative stress plays an important role in the pathogenesis of schizophrenia and MA-induced neurotoxicity (Yamamoto *et al.,* 2010; Chittiprol *et al.,* 2010; Bitanihirwe & Woo, 2011), while SOD activity is impaired in patients with schizophrenia (Raffa *et al.,* 2012). It is therefore noteworthy that MA, but also SIR and MA+SIR significantly reduced SOD activity (Fig. 3-2). Oxidative processes form free radicals such as OH• and O^{-}_{2}• and SOD, a principle antioxidant in the body, effectively scavenges these radicals thereby protecting against oxidative damage (Hussain *et al.,* 1995). Considering our observed 7-fold increase in DA in this brain region, and that hyperdopaminergia is causally related to oxidative stress (Brown *et al.,* 2003; Frost & Cadet, 2000), altered redox state needs to be considered as a basis for our behavioural and monoamine changes. In a recent study, Nakoto *et al* (2011) demonstrated that MA administration increased extracellular glutamate levels, possible correlating with the development of MA-induced decrease in PPI (Nakato *et al.,* 2011; Abekawa *et al.,* 2008; Tata & Yamamoto, 2007). Similarly, SIR-induced deficits in PPI are associated with cortico-striatal oxidative stress (Möller *et al.,* 2001) while SIR also induces changes in cortical glutamate receptors density (Toua *et al.,* 2010). Behavioural deficits observed here may therefore be directly due to abnormalities in glutamate that in turn not only modulates DA release via changes in glutamate-GABA-glutamate activity in cortical pyramidal cells (Stahl, 2007), but can also directly precipitate the development of apoptosis and oxidative stress in the frontal cortex.

It is of interest that the combination of MA+SIR did not evoke an exacerbation of behavioural and neurochemical changes evident in SIR and MA exposed animals separately, especially with evidence that SOD activity tended to show greater loss under this combination (Fig. 3-2). Indeed, reactive adaptive phenomena may be at play to prevent undue redox related changes in DA release and neurotoxicity, with a resultant inhibitory effect on behavioural pathology. One such redox-related regulatory mechanism, for example, involves activating protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). By up-regulating certain genes in response to stress and oxidative stress, these messengers modify cell growth, immunity, inflammation and apoptosis and protect against oxidative stress and its neurobiological consequences (Limón-Pacheco & Gonsebatt, 2009). Such a mechanism may explain why SIR together with MA did not induce statistically significant deficits in regional brain neurochemistry and behaviour.

In conclusion, SIR as well as early life chronic administration of MA in group-reared animals induces profound behavioural changes similar to that seen in schizophrenia, including a decrease in PPI and deficits in outward directed (rearing, approaching, staying together and anogenital sniffing) and an increase in self-directed (self-grooming and squares crossed) behaviours. However, while SIR animals receiving early postnatal MA treatment were similarly

compromised, no obvious exacerbation of these behaviours was observed. Furthermore these behavioural changes occur together with significantly elevated DA levels in the frontal cortex (but not in the striatum) that in turn is associated with oxidative stress, as evidenced by a decrease in frontal cortical SOD activity in MA treated rats, in SIR animals as well as SIR rats receiving MA. Chronic MA administration therefore is capable of inducing psychosis-like bio-behavioural changes to a similar extent as does SIR, a neurodevelopmental animal model of schizophrenia. However, certain adaptive neurophysiological and neuroprotective mechanisms may prevent an exacerbation in pathology in combined SIR+MA treated animals, although further studies in this regard are necessary.

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Chapter 4 – Article 2

The current dissertation is presented in the "article format", as recognised by the North-West University. This chapter presents the core data as a research article prepared for submission to an appropriate peer-reviewed scientific journal. Accordingly, an article titled:

"Effects of prenatal methamphetamine exposure on late-life social interactive and sensorimotor gating behaviours, cortico-striatal monoamines and striatal lipid peroxidation in social and isolation reared rats"

has been prepared as a full-length research report for submission to ***Metabolic Brain Disease.*** Different from other chapters in this dissertation, the current chapter was prepared according to the Instructions to the author for this journal, as described in *Addendum D*. The references for this chapter are therefore provided at the end of the manuscript, and not at the end of the dissertation.

The manuscript is presented in the required format prescribed by *Instructions to the Authors,* as outlined on the journal website:

http://www.springer.com/biomed/neuroscience/journal/11011, under "Instructions for Authors"

The manuscript will begin with the title, contributing authors and affiliations on a separate page, followed by an Abstract also on a single page. Thereafter will follow the main body of the manuscript, including: Introduction, Materials and methods, Results, Discussion and conclusion, Acknowledgements, References, Legends to Tables, Tables, Legends to Figures and Figures. However, for the benefit of the reader, all figures and tables have been included in the text and not at the end of the manuscript as normally required by the Journal.

L. Strauss undertook all the behavioural, neurochemical and statistical analyses, and wrote the first draught of the manuscript. B.H. Harvey designed and supervised the study and assisted in the interpretation of the study data, as well as finalized the manuscript for publication. D.J. Stein and C.B. Brink advised on the study design and proof read the final manuscript.

Title of article

Effects of prenatal methamphetamine exposure on late-life social interactive and sensorimotor gating behaviours, cortico-striatal monoamines and striatal lipid peroxidation in social and isolation reared rats

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Abstract

The abuse of methamphetamine (MA) is increasing under women of childbearing age, with more pregnant women listing MA as their primary drug of choice. Prenatal exposure to MA could have serious consequences on the development of the brain thus laying the foundation for later life bio-behavioural abnormalities that could include an increased susceptibility to psychotic events. Social isolation rearing (SIR) is a valid neuro-developmental animal model of schizophrenia. The current study investigated the effects of prenatal MA exposure *in utero* on behaviour and neurochemical changes later in life, in either group-reared or SIR male Wistar rats. Pregnant Wistar dams were injected subcutaneously (s.c.) for 16 days with 5 mg/kg/day MA or saline (Sal) from prenatal day 13 (PreND-13) to postnatal day 2 (PostND02). On the day of weaning (PostND21) rats were randomly separated into group-reared or SIR groups for 8 weeks. Prepulse inhibition (PPI) and social interaction (SI) was analysed on PostND78 to assess the late-life behavioural effects of prenatal MA exposure. Following behavioural testing rats were sacrificed and cortex and striatum analysed for dopamine (DA), serotonin 5-HT), and noradrenaline (NA), as well as lipid peroxidation in the striatum. SIR significantly reduced outward-directed social behaviours (rearing time, staying together, approaching, anogenital sniffing) and increased self-directed behaviour (self-grooming) but did not affect %PPI. Prenatal MA exposure in group-reared and SIR animals induced similar deficits in outward- and self-directed social behaviours, although no effect on %PPI was observed. No significant differences were observed in brain monoamines between the different groups, although lipid peroxidation was significantly increased in MA treated animals as well as animals exposed to SIR. In conclusion, prenatal MA exposure induces selected behavioural and redox abnormalities akin to schizophrenia in late life, although effects on cortico-striatal monoamines are minimal.

Keywords

Prenatal exposure, social isolation rearing, methamphetamine, Wistar rats, prepulse inhibition, social interaction, striatum, frontal cortex, monoamines, lipid peroxidation, oxidative stress.

4.1 Introduction

Methamphetamine (MA), a central nervous system (CNS) stimulant, has potential neurotoxic effects on the developing brain, especially involving monoaminergic systems most notably the dopamine (DA) system (Arria *et al.,* 2006). Of growing concern is that many women of childbearing age who are substance abusers list amphetamines as their primary drug of abuse (Piper *et al.,* 2011; Grace *et al.,* 2010). MA crosses the placental barrier, exposing the developing foetus to its toxic effects (Bubenikova-Valesova *et al.,* 2009). Dopaminergic systems begin to form as early as the first trimester, so that prenatal exposure to MA could have serious consequences on the development of the brain (McFadden *et al.,* 2011). As a result, there is an increased risk for bio-behavioural abnormalities later in life that would include an increased susceptibility to psychotic events.

Prenatal MA exposure in animals induces numerous abnormalities in offspring, including delayed motor development, increased maternal mortality and decreased growth (Arria *et al.,* 2006). In humans with a history of prenatal MA exposure, behavioural abnormalities (Arria *et al.,* 2006) as well as long term cognitive deficits have been documented (Bubenikova-Valesova *et al.,* 2009). Behavioural abnormalities that can develop later in life include a failure to adjust in a social environment, aggressive behaviour and poor performance on psychometric tests (Frost & Cadet, 2000), while these neurobehavioural deficits may persist well into adulthood (Cruickshank & Dyer, 2009). *In utero* exposure to MA can also result in long-term detrimental effects, including CNS birth defects and abnormalities in emotional development (Piper *et al.,* 2011). Poor performance in attention and memory tests has also been described in children exposed to MA *in utero* (Vearrier *et al.,* 2012).

MA impacts on multiple neurotransmitter systems, affecting DA, serotonin (5-HT) and noradrenalin (NA) (Vearrier *et al.,* 2012; Yamamoto & Raudensky, 2008). MA is also a potent releaser of these monoamines, increasing their concentrations in the synaptic cleft (Kirkpatrick *et al.,* 2012; Yamamoto *et al.,* 2010). Of note is that a number of studies have shown the relationship between MA and oxidative stress. Lipid peroxidation products are increased after MA exposure (Yamamoto *et al.,* 2010), with increased malonyldialdehyde observed in the hippocampus and striatum of rats (Tata & Yamamoto, 2007).

Schizophrenia is a devastating psychiatric disorder affecting around 1% of the world's population (Nilsson *et al.,* 2005), with many factors hypothesised to contribute to its development (see Seeman, 2011 for review). Early-life neurodevelopmental damage is one of the foremost factors thought to contribute to the development of schizophrenia (Daenen *et al.,* 2003). Such neurodevelopmental changes, originating for example from childhood trauma, isolation, psychosocial stress and smoking, are purported to increase vulnerability for developing schizophrenia (Seeman, 2011). Chronic, high dose abuse of MA may lead to the

development of psychiatric symptoms referred to as MA psychosis (Nakamura *et al.,* 2009; Yui *et al.,* 2000; Srisurapanont *et al.,* 2011), in most cases indistinguishable from schizophrenia (Cruickshank & Dyer, 2009; Yui *et al.,* 2000).

Animal models of early life adversity can aid in better understanding the underlying mechanisms involved in MA abuse and vulnerability to developing psychosis later in life (Dimatelis *et al.,* 2012). Post-weaning social isolation rearing (SIR) of rodents reproduces many of the typical behavioural and neurochemical responses of schizophrenia (Fone & Porkess, 2008). In fact drug abuse and social isolation are two important non-genetic factors that contribute towards the development of schizophrenia (Seeman, 2011).

The current study investigated the effects of prenatal MA exposure in rats on behaviour later in life, as well as changes in cortico-striatal levels of DA, 5-HT and NA and lipid peroxidation. Thereafter we studied whether these bio-behavioural responses are exacerbated in a population of already vulnerable animals, viz. SIR animals.

4.2 Materials and methods

4.2.1 Animals

Pregnant female Wistar dams, as carriers of the unborn foetuses, were used during the prenatal phases of the study for the administration of MA or vehicle. We estimated that a pregnant dam would produce 4 or more male pups that would be used in the study, although sex distribution varied. At weaning (PostND21) the pre-selected male rats (projected n=10/group) were randomly separated into group-reared animals (groups of 3-4 rats/cage) and SIR animals (1 rat/cage) for 8 weeks (PostND77). Adult rats weighed 250 g – 300 g on the day of behavioural testing and were reared in identical cages containing sawdust. All animals were housed under conditions of constant temperature (22 ± 1℃) and h umidity (50 ± 10%) with a 12:12-h light/dark cycle (lights on 06:00 to 18:00). Food and water were provided ad libitum. During the isolation period, animals were maintained in an environment with no enrichment and minimal handling. Cages were changed once a week with fresh sawdust.

The study and all animal procedures were approved by the Ethics Committee of the North-West University (*approval number: NWU-000105-11-S5*) and were in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

4.2.2 Drug treatment

Methamphetamine hydrochloride (MA) was dissolved in saline and administered to the pregnant dams via sub-cutaneous (s.c.) injection at a dose of 5mg/kg/day (Yamamotová *et al.,* 2011; Bubenikova-Valesova *et al.,* 2009; Slamberová *et al.,* 2006). Dosing took place between 9:00

and 10:00 each morning for 16 consecutive days from PreND-13 to PostND02. Saline was used as vehicle control. Pregnant dams were weighed every day and dosed according to weight on that specific day. On PostND21, male rat pups were weaned from their mother and divided into group-reared or SIR groups (6 rats/group), as described above. All animals were sacrificed by decapitation without any prior use of an anesthetic agent.

4.2.3 Behavioural tests

After weaning on PostND21 the animals were reared under either group or SIR housing conditions until PostND78, at which time the behavioural tests described below were performed. All behavioural tests were performed sequentially between 1 to 4 hours after the start of the dark cycle (i.e. 19:00 – 22:00).

Prepulse inhibition (PPI) testing

Schizophrenia typically presents with deficits in sensorimotor gating that can be assessed by determining %PPI (Powell *et al.,* 2009). PPI was assessed in two illuminated and ventilated sound–attenuated startle chambers (SR-LAB, San Diego Instruments, San Diego, USA; Van den Buuse *et al.,* 2003; Powell *et al.,* 2009) recently set up and validated in our laboratory (Möller 2009; Möller *et al.,* 2011). A stabilimeter system composed of a transparent Plexiglas cylinder (diameter 8.2 cm, length 20 cm) mounted on a Plexiglas frame is present in each startle chamber. The acoustic noise bursts are provided by a speaker mounted 24 cm above the cylinder. A piezoelectric accelerometer mounted below the frame detects and transduces the startle responses of the rat within the cylinder. Stimuli are delivered and startle responses measured during each session using SR-Lab software (San Diego Instruments) running on a Hewlett-Packard personal computer. Startle amplitudes were defined as the average of one hundred 1-ms stabilimeter readings collected from the stimulus onset. The stabilimeter was calibrated before each test session.

An identical protocol, adapted from previously described methods (Wang *et al.,* 2003; Möller, 2009; Möller *et al.,* 2011), was used for all experiments. The startle session starts with a 5-min acclimatization period, with a 68-dB background noise level that continues throughout the test session, followed by four blocks of trials with either pulse, no-pulse or prepulse trials, with a total of 100 trials (see Möller *et al.,* 2011). % PPI for the four prepulse intensities (72, 76, 80 and 84 dB) used was calculated according to the formula: % PPI = [(Pulse-alone amplitude – Prepulse + pulse amplitude)/Pulse-alone amplitude] x 100 (Ettinger *et al.,* 2005; Cadenhead, 2011). The last 10 trials consist of single 40 ms 120 dB pulse-alone startle stimuli. The total of 100 trials was delivered with an average interval of 25 s. The first and last 10 pulse-alone stimuli (BLOCK 1 and BLOCK 4, respectively) and the 20 pulse-alone stimuli included in the PPI block itself (BLOCK 2 and BLOCK 3), were used to obtain a measure of mean startle amplitude suggestive

of habituation in response to repeated delivery of startling stimuli (Wang *et al.,* 2003; Van den Buuse *et al.,* 2003; Möller, 2009).

Social interaction test

The SI test is used to assess spontaneous social interactive (outwardly directed) and self (inwardly)-directed behaviours in rodents using the open field test (OFT) (Shimazaki & Chaki, 2005; Ferdman *et al.,* 2007), the latter being well-described behavioural manifestations of schizophrenia (Powell *et al.,* 2009). This procedure has been approved by the North-West University Ethical Committee (NWU-0035-08-S5), and has been established in our laboratory (Möller 2009; Möller *et al.,* 2011). This test is also performed as a measurement of spontaneous locomotor activity in rodents (Gonzalez *et al.,* 1996; Ferdman *et al.,* 2007). The apparatus used in this test consists of a black square (70 cm x 70 cm x 40 cm) arena, divided into 35 cm x 35 cm equal squares drawn onto the floor of the arena (Sherif & Oreland, 1994). The arena is illuminated with red light (40 W), with a digital video camera mounted above the arena to record all spontaneous behaviours.

Two rats were placed in the centre of the floor and allowed to adapt for 30s. One of the two rats was tested for social interaction with an unknown test partner that did not differ by more than 10g in weight. Both members of a test-pair received the same treatment as well as the same prior experience. The time spent in active social interactive behaviours, including rearing, approaching and staying with the partner and anogenital sniffing (Gonzalez *et al.,* 1996), as well as self-directed behaviour, including self-grooming and squares crossed (Gonzalez *et al.,* 1996), was scored over a period of 10 min. Subsequent video analyses was then performed and scored. Testing was performed between 19:00 – 22:00, with the researcher remaining outside the testing room during the 10 min testing session. After each test the arena was cleaned with 10 % ethanol solution to remove urine, faeces and odour trails (Ferdman *et al.,* 2007).

4.2.4 Neurochemical analysis

Preparation of brain tissue

To avoid undue effects of behavioural testing on the neurochemical analyses performed, rats were sacrificed by decapitation 24 hours after the final behavioural test and the frontal cortex and striatum immediately dissected out on an ice-cooled glass slab. After removing the olfactory bulb from the cortex and then cutting around the anterior tip of the corpus callosum, the frontal cortical tissue was dissected (Möller, 2009; Toua *et al*., 2009). When dissecting the striatum, the dorsal side of the brain was placed side up. Then the two cerebral hemispheres were split and the striatum dissected with the corpus callosum as external limits and the external walls of the lateral ventricles as internal limits. Immediately afterwards the striatum and frontal cortex

were fixed in liquid nitrogen and stored in the -80℃ freezer until the day of assay. On the day of assay, the striatum and frontal cortex were removed from the freezer, weighed and allowed to thaw on ice.

Regional brain monoamine analysis

Frozen brain tissue collected after sacrifice was used for regional quantification of DA, 5-HT and NA in the frontal cortex and striatum using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (Harvey *et al.,* 2006; 2010). Monoamine concentrations in the samples were determined by comparing the area under the peak of each monoamine in the sample to that of the monoamine standard (isoprenaline, range 5–50 ng/ml; Chemstation Rev. A 06.02 data acquisition and analysis software). Linear standard curves (regression coefficient greater than 0.99) were found in this range. Monoamine concentrations were expressed as ng/mg wet weight of tissue (mean ± SEM). The limit of quantification (LOQ) for the monoamine assay is 10 ng/ml. The limit of detection (LOD) is 2.5 ng/ml (Harvey *et al.,* 2006).

Analysis of lipid peroxidation

Since increased lipid peroxidation has been described specifically in the striatum of MA-treated animals (Tata & Yamamoto, 2007), and that the amount of brain tissue available was too little for assay of lipid peroxidation in both frontal cortex and striatum, we only analysed lipid peroxidation in the striatum in an attempt to demonstrate cell damage in the brain following the different rearing conditions with/without MA exposure.

To quantify the degree of lipid peroxidation in tissue, a commercially available kit (Lipid Peroxidation Assay Kit®, BioVision, Centurion, South Africa) was used. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are natural end products of lipid peroxidation and the measuring of these components is a widely accepted method to determine oxidative stress. Immediately prior to MDA determination, the frozen brain tissue was thawed on ice and weighed. MDA reacts with thiobarbituric acid (TBA) to generate the MDA-TBA product that can be quantified colorimetrically. The absorbance of the latter was then determined at 532nm using a multiscan plate reader (AEC Amersham).

4.2.5 Statistical analysis

Multiple comparisons relative to control were analysed by two-way analyses of variance (ANOVA) followed by the Tukey post-test. The unpaired Student's t-test (two-tailed) was performed to compare the data from two treatment groups. GraphPad Prism® (version 5.00, San Diego California, U.S.A.) was used for all statistical analyses and graphical representation. Data are presented as averages ± S.E.M. with a value of $p<0.05$ taken as statistically significant.

4.3 Results

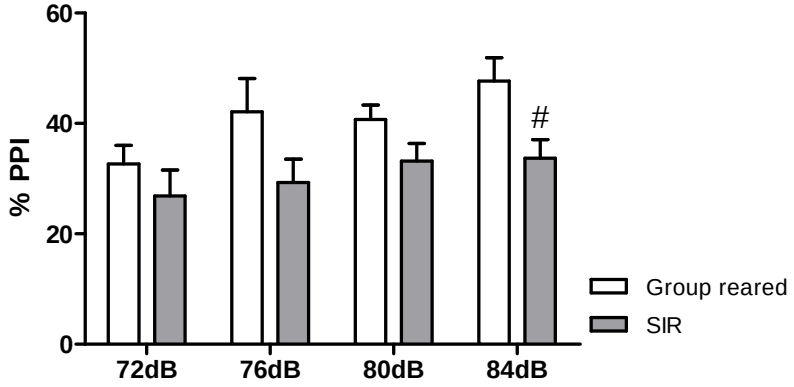
4.3.1 Behavioural studies: Prepulse inhibition (PPI)

Two-way ANOVA revealed no significant PPI*housing condition interaction across the groups (Fig. 4-1A). Indeed %PPI was not significantly altered in SIR rats compared to their group-reared controls, with the only significant effect observed being a reduction in %PPI at a prepulse intensity of 84 dB ($p<0.03$).

In the drug treatment study two-way ANOVA similarly found no significant interactions in group-reared and SIR animals regarding %PPI*treatment, %PPI*housing condition and treatment*housing condition (Fig. 4-1B). Post hoc Tukey tests revealed a significant difference at 84 dB between group-reared saline animals and group-reared animals receiving MA ($p<0.050$) and SIR animals receiving MA treatment ($p<0.05$) (Fig. 4-1B). The suppression of %PPI at 84 dB induced by SIR as noted earlier was now lost. None of treatment-rearing groups differed with respect to one another (other than vs. group-reared saline).

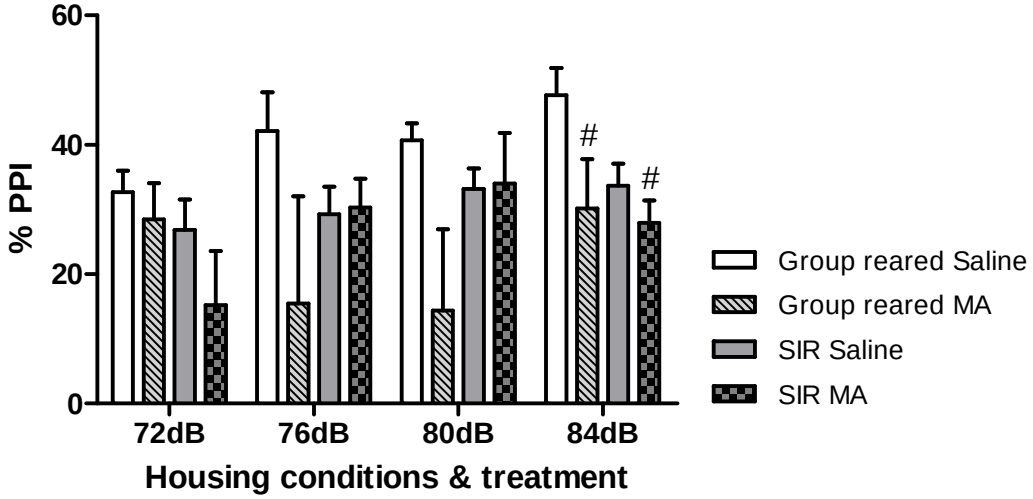
Figure 4-1

A



Statistics: #p<0.05 vs. group-reared (Student's t-test (two-tailed))
see literature for precise p values

B



Sensorimotor gating at prepulse intensities as indicated, in group-reared and SIR rats in the drug treatment cohort (n=6/group), as determined by percentage prepulse inhibition (%PPI). The unpaired Student's t-test (two-tailed) was performed to compare the data of two treatment groups in the non-treatment cohort. In the treatment cohort a two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of p<0.05 was taken as statistically significant. Statistics: #p<0.05 vs. group-reared saline (Two-way ANOVA, Tukey) at 72 dB, 76 dB, 80 dB and 86 dB respectively

4.3.2 Behavioural studies: Social interaction studies

The saline-treated cohort vs. grouped-reared controls (data not shown) was incorporated into the drug-treated cohort for analysis. Two-way ANOVA revealed significant cross-group interactions in the drug treatment cohort with respect to social interactive behaviours, viz. time

spent rearing ($F(1,20)=12.238$, $p=0.002$), approaching ($F(1,20)=8.006$, $p=0.01$), staying together ($F(1,20)=6.501$, $p=0.02$) and anogenital sniffing ($F(1,20)=9.413$, $p=0.006$) (Table 4-1).

Rearing: Post hoc analysis with Tukey revealed a significant decrease in rearing in SIR animals receiving saline ($p=0.02$) and the SIR animals receiving MA ($p<0.001$) compared to the group-reared control animals receiving saline (Table 4-1). Group-reared animals receiving MA was numerically lower but missed significance.

Approaching: A significant decrease in approaching was noted in group-reared animals receiving MA ($p=0.007$), in SIR animals receiving saline ($p<0.005$) and in SIR animals receiving MA ($p=0.02$) compared to the group-reared control animals receiving saline (Table 4-1).

Staying together: Staying together time for the group-reared animals receiving MA ($p=0.02$), SIR animals receiving saline ($p=0.02$) and the SIR animals receiving MA ($p=0.007$) was significantly decreased compared to the group-reared control animals receiving saline (Table 4-1).

Anogenital sniffing: This parameter was significantly decreased in group-reared animals receiving MA ($p=0.003$), SIR animals receiving saline ($p=0.002$) and in SIR animals receiving MA ($p=0.006$) compared to the group-reared control animals receiving saline (Table 4-1). In all of the above behaviours, none of the treatment-rearing groups differed with respect to one another (other than vs. group-reared saline).

When considering self-directed behaviours, two-way ANOVA revealed a significant cross-group interaction for self-grooming ($F(1,20)=17.780$, $p<0.001$), but no significant interaction for squares crossed ($F(1,20)=0.005$, $p=0.945$) (Table 4-1).

Self-grooming: Post hoc analysis with Tukey revealed a significant increase in self-grooming for group-reared animals receiving MA ($p<0.03$), SIR animals receiving saline ($p<0.002$) and SIR animals receiving MA ($p<0.001$), compared to the group-reared-control animals receiving saline (Table 4-1).

Squares crossed: No significant changes with respect to squares crossed was evident with in any of the groups tested compared to the group-reared control animals receiving saline (Table 4-1). In all of the above behaviours, none of treatment-rearing groups differed with respect to one another (other than vs. group-reared saline).

Table 4-1: Behavioural analysis in group-reared or SIR animals receiving either saline or MA

Parameter measured	GR Saline Mean ± SEM	GR MA Mean ± SEM	SIR Saline Mean ± SEM	SIR MA Mean ± SEM
Behaviour:				
Social interaction				
Rearing (s)	34.83 ± 1.92	24.167 ± 3.35	21.33 ± 3.53 #	15.5 ± 2.26 **
Approaching (s)	11.83 ± 1.01	6.5 ± 0.56 *	6.33 ± 1.59 *	7.17 ± 0.95 #
Staying together (s)	11.50 ± 1.73	5.167 ± 1.11 #	4.83 ± 1.94 #	4.00 ± 1.21 *
Anogenital sniffing (s)	19 ± 1.29	10.33 ± 1.59 *	9.83 ± 2.34 *	11.00 ± 0.78 *
Self-interaction				
Self-grooming (s)	2 ± 0.63	4.167 ± 0.31 #	5.00 ± 0.68 *	5.67 ± 0.42 **
Squares crossed	124.83 ±7.65	115.67 ± 9.85	140.00 ± 11.36	132.33 ± 13.1

Social interactive and self-directed behaviours in group-reared and SIR rats in the treatment cohort (n=6/group): time spent rearing, time spent approaching, time spent together, time spent anogenital sniffing, time spent self-grooming and squares crossed (locomotor activity). A two-way ANOVA followed by a Tukey post-test was performed to compare the different drug treatment groups with their respective control. All data is presented as averages ± S.E.M. with a value of $p<0.05$ taken as statistically significant. Statistics: **$p<0.001$ vs. group-reared saline (Two-way ANOVA, Tukey), *$p<0.01$ vs. group-reared saline (Two-way ANOVA, Tukey), #$p<0.05$ vs. group-reared saline (Two-way ANOVA, Tukey). GR – Group-reared; SIR – Social isolation reared; MA –methamphetamine

4.3.3 Neurochemical studies

Regional brain monoamines

The saline-treated cohort vs. grouped housed controls (data not shown) was incorporated into the drug-treated cohort for analysis. Two-way ANOVA revealed no significant cross-group interactions in the frontal cortex or striatum of the drug treatment cohort (Table 4-2). No significant difference in DA, 5-HT or NA was observed in either the frontal cortex or striatum in any of the groups tested compared to their group-reared controls or with each other (Table 4-2).

Table 4-2: Regional brain monoamines in group-reared and SIR animals receiving either saline or MA

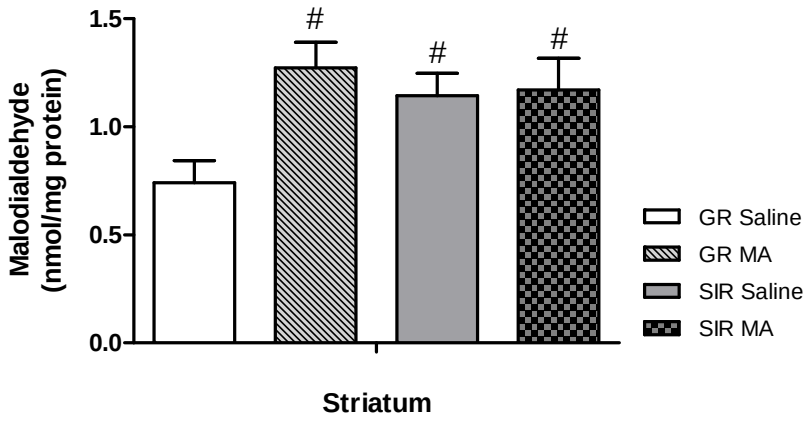
Parameter measured	GR Saline Mean ± SEM	GR MA Mean ± SEM	SIR Saline Mean ± SEM	SIR MA Mean ± SEM
Monoamines				
Frontal cortex				
NA	195.13 ± 8.87	226.98 ± 8.87	210.71 ± 9.71	230.26 ± 8.87
DA	79.60 ± 136.16	369.01 ± 136.16	168.92 ± 149.16	537.44 ± 136.16
5-HT	170.69 ± 15.45	198.8 ± 15.45	186.57 ± 16.92	231.79 ± 15.45
Striatum				
NA	174.71 ± 52.65	288.17 ± 52.65	227.52 ± 57.68	259.20 ± 52.65
DA	3402.75 ± 321.55	3600.42 ± 321.55	3335.10 ± 352.24	3513.22 ± 321.53
5-HT	283.71 ± 40.05	402.94 ± 40.05	343.56 ± 43.87	408.94 ± 40.05

Frontal cortex and striatum monoamines in group-reared and SIR rats in the treatment cohort (n=6/group): NA, DA and 5-HT. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. All data is presented as averages ± S.E.M. with a value of $p < 0.05$ taken as statistically significant. GR – Group-reared; SIR – Social isolation reared; MA – methamphetamine

Lipid peroxidation

The saline-treated cohort vs. grouped-reared controls (data not shown) was incorporated into the drug-treated cohort for analysis. In the drug treatment cohort (Fig. 4-2), two-way ANOVA analysis revealed the following significant interactions: treatment*brain region ($F(1,19)=5.307$, $p=0.03$) and treatment*housing condition ($F(1,19)=4.332$, $p=0.05$). Post hoc analysis with Tukey showed a significant elevation of malondialdehyde in the striatum of the group-reared animals receiving MA ($p=0.01$), SIR animals receiving saline ($p=0.05$) as well as SIR animals receiving MA ($p=0.039$) (Fig. 4-2). However, none of treatment-rearing groups differed with respect to one another (other than vs. group-reared saline).

Figure 4-2



Concentration of malondialdehyde, a measurement of lipid peroxidation, in the striatum of socially reared and SIR rats (n=6/group) in the drug treatment cohort. A two-way ANOVA followed by a Tukey post-test was performed to compare the different drug treatment groups with their respective control. A value of $p<0.05$ was taken as statistically significant. Statistics: #$p<0.05$ vs. group-reared saline (Two-way ANOVA, Tukey)

4.4 Discussion

The most important observations of this study are that SIR significantly reduced outward-directed social behaviours (rearing time, staying together, approaching, anogenital sniffing) and increased self-directed behaviour (self-grooming) although it did not affect %PPI. Prenatal MA exposure in group-reared and SIR animals significantly reduced outward-directed behaviours, increasing self-directed social behaviours but also did not significantly affect %PPI. No significant differences were observed in brain monoamines between the different groups, although lipid peroxidation was significantly increased in MA, SIR and MA+SIR treated animals.

The abuse of MA has increased under pregnant women, exposing the growing foetus to the pharmacologic and toxic effects of the drug and its metabolites (Piper *et al.,* 2011; Grace *et al.,* 2010; Heller *et al.,* 2001a; Vearrier *et al.,* 2012). Exposure to MA can adversely affect the development of the CNS (Piper *et al.,* 2011) resulting in neurobehavioural deficits that may persist well into adulthood (Cruickshank & Dyer, 2009). Prenatal exposure to MA is also associated with a greater risk of developing behavioural abnormalities later in life, including aggression, failure to adjust in a social environment and poor performance on psychometric tests (Frost & Cadet, 2000). Little is known about the effects of MA exposure *in utero* on the development of late-life abnormalities. The aim of this study was to determine if *in utero* MA exposure predisposes normal (group-reared) or vulnerable (SIR) individuals to develop psychotic-like behavioural manifestations later in life.

SIR is well known to induce behavioural changes related to schizophrenia (Fone & Porkess, 2008). Indeed, previous work in our laboratory (Möller *et al.,* 2011), by others (Geyer *et al.,* 2001; Powell *et al.,* 2009; Broberg *et al.,* 2010), as well as studies undertaken in Chapter 3, have confirmed the ability of SIR to attenuate sensorimotor gating and social interactive behaviours in rodents. Although SIR alone did indeed significantly attenuate social (rearing, approaching, staying together, anogenital sniffing) and self-directed (self-grooming) behaviours in this study (Table 4-1), it failed to alter %PPI vs. group-reared controls (Fig. 4-1). Due to complications in attaining the appropriate numbers of male pups at birth, the resulting small sample of animals/group (6 rats/group) could have adversely affected the results. Nevertheless, with at least some inhibitory effect evident at 84 dB (Fig. 4-1A), as well as the reduction of social- and increase of self-directed social behaviours (Table 4-1), these data still attests to the face validity of the model.

As with the SIR group alone, we observed no significant differences in %PPI in the MA exposed group, except at 84 dB (Fig. 4-1B), suggesting that MA exposure *in utero* did not induce noteworthy deficits in sensorimotor gating later in life. However, earlier studies (Grant *et al.,* 2011) as well as studies undertaken in Chapter 3 confirm that MA can indeed adversely affect sensorimotor gating in adolescent and adult animals (not exposed *in utero*). This and the lack of response of SIR noted above, an otherwise widely regarded robust model of schizophrenia, raises concerns that conditions may not have been optimal in the study. Indeed, while we have previously shown SIR to change *all* social interactive behaviours (Möller *et al.,* 2011; current study, Chapter 3), here SIR failed to significantly alter squares crossed (Table 4-1). Indeed, despite the well-controlled nature of the study, it is nevertheless possible that certain extraneous factors could have adversely affected the study, possibly underlying these less than emphatic results. This is discussed later.

Prenatal exposure to MA nevertheless significantly affected social interaction compared to saline-treated group-reared controls (Table 4-1). Social interactive behaviour (approaching, staying together and anogenital sniffing) was significantly decreased and self-directed behaviour (self-grooming) was significantly increased. It has been proposed that these behaviours resemble the positive and negative symptoms observed in schizophrenia, respectively (Fone & Porkess, 2008; Möller *et al.,* 2011), confirming that MA did indeed provoke the development of psychotic-like behaviours in late life. However MA is anxiogenic, having been found in most studies to induce anxiety in animals (Schutová *et al.,* 2009). The observed decrease in social interaction can therefore also be linked to the anxiogenic effects of the drug (Schutová *et al.,* 2009), although PPI is not affected by anxiety, explaining why no differences in PPI was observed. However, we did not observe any changes in squares crossed, an indication of locomotor activity, which is usually reduced in anxious states. Moreover, rats prenatally exposed to MA have been found to spend more time freezing, thus presenting with a reduction

in locomotor activity (Schutová *et al.,* 2009) and in line with our findings. Of note is that abnormalities in locomotor activity induced by MA are related to changes in brain monoamine transmission (DA, 5-HT and NA) (Tan, 2003). However, since we did not observe any changes in cortico-striatal monoamines, indicates that prenatal MA exposure failed to alter late-life locomotor activity in this study.

Studies have shown that although DA levels are elevated in the unborn foetus during prenatal MA exposure, in adulthood the offspring show normal levels of DA and 5-HT (Heller *et al.,* 2001a), which is congruent with our findings. Nevertheless, a disruption in dopaminergic function at such an early stage can still influence behaviour that persists into adulthood (Heller *et al.,* 2001a). So while we observed no significant differences in brain monoamines 11 weeks after prenatal exposure, significant behavioural abnormalities were nevertheless present at this time, viz. social interaction, indicating that the disruption of DA function *in utero* did indeed affect the behaviour of the animals in adulthood. Unfortunately, we were not able to study the levels of DA shortly after prenatal MA exposure to confirm this.

It is pertinent, however, to point out that most behavioural studies following after *in utero* MA exposure determine the resultant effects on behaviour and PPI in adulthood immediately after a second MA challenge (Tan, 2003; Schutová *et al.,* 2010; Bubenikova-Valesova *et al.,* 2009). These authors essentially demonstrate how *in utero* MA exposure may sensitize the individual in later life to developing behavioural abnormalities. However, we exposed rats to MA *in utero* with *no* additional administration in adulthood or immediately prior to behavioural testing. Behavioural changes, such as deficits observed in PPI, are closely related to monoamine disruptions (Tan, 2003) that can be observed more distinctly after acute administration of MA. Concurrent with previous studies (Heller *et al.,* 2001a), we did not observe any significant changes in monoamines in adulthood in rats exposed *in utero*. This may explain why no deficits in PPI were observed, confirming that MA exposure *in utero* does not induce deficits in sensorimotor gating later in life. It would have been of interest to see if this picture changes following re-challenge with MA in later life, as described by other authors (noted above).

Alternatively, it may imply that early MA exposure impacted on non-monoamine neurotransmitter systems responsible for these behavioural abnormalities. A study in children exposed to MA *in utero* found increased concentration of glutamate (GLU) in the striatum, suggesting an increase in cellular metabolic activity that may persist for years after exposure (Williams *et al.,* 2004). The glutamatergic system has been implicated in the pathophysiology of schizophrenia and MA-induced neurotoxicity (Frost & Cadet, 2000). Moreover, MA has been shown to increase extracellular concentrations of GLU in the striatum (Tata & Yamamoto, 2007) contributing to the development of oxidative stress and possibly behavioural abnormalities.

Unfortunately GLU was not determined in this study and therefore similar conclusions cannot be drawn. Nevertheless, this represents a valuable avenue of investigation for future studies.

Finally, the increase in lipid peroxidation evoked by MA, SIR and MA+SIR (Fig. 4-2) highlights the critical role that cell damage plays in the bio-behavioural response to early life stress, which can be either a psychosocial stressor such as SIR or a chemical agent such as MA. Earlier studies have noted an increase in oxidative stress and lipid peroxidation following SIR in rats (Möller *et al.,* 2011), while oxidative stress and apoptosis are intimately involved in MA-associated brain damage (Brown *et al.,* 2003; Vearrier *et al.,* 2012; Riddle *et al.,* 2006). The developing foetus lacks most antioxidant enzymes, making it vulnerable to drug-induced oxidative stress (Jeng *et al.,* 2005). In a study done by Heller *et al* (2001b) it was observed that prenatal MA exposure resulted in an enhanced neurotoxic risk in adulthood. In the current study we observed an elevation in malondialdehyde levels, a product of lipid peroxidation, in the striatum of MA treated animals, consistent with findings that high levels of MA in the striatum during *in utero* exposure increases the susceptibility of this region for MA-induced neurodevelopmental abnormalities and oxidative stress (Jeng *et al.,* 2005). However, while SIR and MA separately induced an increase in striatal lipid peroxidation, their effects were not additive in the combined MA+SIR treated animals. It is interesting that MA+SIR induced a greater elevation in frontal cortical DA than either MA or SIR alone (Table 4-2), although this was not significant. This increase in DA and its association with oxidative stress was also observed in our earlier postnatal MA study (Chapter 3). An increase in DA turnover is widely regarded as a major contributor to cellular oxidative stress and damage. The absence of an additive effect in SIR + MA exposed animals on lipid peroxidation is interesting. However, as mentioned in my previous manuscript (Chapter 3), activating protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are implicated in the upregulation of certain genes in response to stress and oxidative stress and are central messengers commissioned with the function of protecting the cell against the ravages of oxidative stress (Limón-Pacheco & Gonsebatt, 2009). This natural protective mechanism could explain why SIR together with MA did not induce a statistically significant increase in malondialdehyde in MA + SIR treated animals.

Although this study has important heuristic value, evidence provided by some of the behavioural data suggests that the conditions of study may not have been optimal. Although every attempt at maintaining strict control over extraneous stressors known to influence the behavioural and physiological response of animals, such as controlling for injection stress and arrangement of the behavioural test battery with the acoustic startle last (the more stressful test), environmental stress may still have exerted an unexpected influence, especially with respect to SIR effects on PPI. Placing animals in an unfamiliar environment is regarded as a painless psychological stressor but is often accompanied by changes in brain monoamines such as DA, 5-HT and NA

(Miura *et al.,* 2002). Although the more stressful test was placed last, viz. acoustic startle, placing rats in the open field test and then in the acoustic startle apparatus, both unfamiliar environments and without a period of adaptation, may have engendered a stress response to the novel environment. Although injection was controlled for, the effect of stress on the dam should also be taken into consideration. Dams were injected daily for 16 days during pregnancy and could be considered a stressful experience. Since individual animals may experience the stressor differently, this may introduce variable effects of stress hormones on the foetus that is difficult to control for. Indeed, maternal stress during pregnancy produces signs of long-term behavioural abnormalities in the offspring, including anxiety and depressive like symptoms (Maccari *et al.,* 2003). This could adversely affect the results obtained in the study and future studies should consider alternative methods of administration, i.e. orally through drinking water.

In conclusion, SIR proved partially effective in inducing aberrant social interactive behaviours related to schizophrenia, although failed to affect sensorimotor gating. Prenatal MA exposure also induced deficits in social interaction but not in PPI, while neither notably affected regional brain monoamines. However increased lipid peroxidation was evident in the striatum in MA, SIR and MA+SIR treated animals, indicating the presence of cellular damage, possibly of redox origin, although no additive effect was observed in MA+SIR animals. We can therefore conclude that prenatal MA exposure does indeed induce bio-behavioural abnormalities akin to schizophrenia in late life, indicating that prenatal MA exposure can predispose certain susceptible individuals to developing psychotic-like manifestations later in life. However, an additive effect in MA + SIR animals was not evident. This work warrants further study to confirm its findings.

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Chapter 5

Summary, conclusion and recommendations

Since the results of the current project are presented in three separate sections, namely Chapter 3 (Article 1), Chapter 4 (Article 2) and Addendum B (Additional results), it is necessary to provide a concise summary of all the results as well as a brief discussion on the impact of these findings and their relevance to the field. This will allow an appropriate conclusion to be made, together with recommendations for future studies.

This chapter represents results obtained from various behavioural tests performed on postnatal day 78 (PostND78) in pre- or postnatal methamphetamine (MA) exposed group- or social isolation-reared (SIR) animals. Thereafter post-rearing analysis of monoamine and redox related changes in the frontal cortex and striatum were performed. The methods regarding the preparation and performing of specified behavioural and neurochemical analyses can be found in Chapter 3 (Article 1) and Chapter 4 (Article 2) while background information on these tests can be found in Addendum A (Materials and methods) and Addendum C (Neurochemical analysis).

The study aimed to address the following study objectives, as outlined in Chapter 1 (§1.3):

- Determine whether chronic early life postnatal exposure to MA may sensitize normal (group-reared) or predisposed (SIR housed) individuals to the development of schizophrenia-like behaviours later in life.
- Determine whether chronic prenatal (*in utero*) exposure to MA may sensitize normal (group-reared) or predisposed (SIR housed) individuals to the development of schizophrenia-like behaviours later in life.
- Determine schizophrenia like behaviour using the prepulse inhibition (PPI) and social interaction tests to determine deficits in sensorimotor gating and social- and self-directed behaviours, respectively.
- Investigate a possible correlation between the observed behavioural changes in the animals with respect to cortico-striatal brain monoamine levels and markers of oxidative stress.
- Compare group-reared rats to SIR rats in order to determine if prior adverse experience (SIR) represents a risk factor to developing more severe behavioural abnormalities following MA exposure.

- ❖ Investigate post-mortem changes in regional brain monoamines, superoxide dismutase activity (SOD) and lipid peroxidation in SIR vs. group-reared animals following either prenatal or postnatal MA exposure.

5.1 Summary of study outcomes

5.1.1 Postnatal MA Study

1. I demonstrated that chronic early postnatal administration of MA induced significant deficits in PPI in later life
2. I observed that chronic MA resulted in significantly decreased social interactive behaviours, viz. rearing, approaching, staying together and anogenital sniffing, as well as significantly increased self-directed behaviours, viz. self-grooming and squares crossed. These same behavioural changes were also induced in the SIR animals receiving saline.
3. I noted that group-reared animals receiving MA and SIR animals receiving saline showed a significant increase in DA in the frontal cortex, with no other monoamines were affected in the frontal cortex (NA, 5-HT) or striatum (NA, DA, 5-HT). Regarding the metabolites of these monoamines (MHPG, DOPAC, HVA and 5-HIAA) in the frontal cortex, I observed significant decrements in MHPG in SIR animals receiving MA as well as increased DOPAC and HVA in the group-reared animals receiving MA. There were no significant changes observed in 5-HIAA. In the striatum I observed a significant decrease in MHPG in MA treated animals, but no significant differences in the other metabolites.
4. I observed a significant decrease in SOD activity in the frontal cortex of MA treated animals as well as SIR animals receiving saline.
5. With respect to bio-behavioural changes induced by either MA or SIR alone, I observed no additive effects in any of these parameters in SIR animals receiving MA.

5.1.2 Prenatal MA Study

1. In the prenatal study I found that *in utero* exposure to MA, or SIR alone, did not induce deficits in PPI.
2. I observed deficits in social interactive behaviours, viz. approaching, staying together and anogenital sniffing, and increased self-directed behaviours, viz. self-grooming in group-reared animals receiving MA. These same behavioural changes were also induced in the SIR animals receiving saline.
3. Regarding regional brain monoamines I found no significant differences in NA, DA, 5-HT or their metabolites in the frontal cortex or striatum.

4. I observed in increase in malondialdehyde, a product of lipid peroxidation, in the striatum of animals receiving MA as well as SIR animals receiving saline.
5. With respect to bio-behavioural changes induced by either MA or SIR alone, I observed no additive effects in any of these parameters in SIR animals exposed to MA *in utero*

5.2 Conclusion

In the postnatal study I observed significant abnormalities in PPI and social interaction in both the MA treated groups as well as the SIR group receiving saline. The significant reduction in %PPI in these animals confirms that MA abuse during adolescence induces profound sensory motor gating deficits similar to that seen in a neurodevelopmental animal model of schizophrenia, viz. SIR animals. Furthermore MA administration significantly affected social interaction, decreasing social interactive behaviours and increasing self-directed behaviours, again with a severity matching that induced in SIR animals. These behavioural deficits are closely linked to the positive and negative symptoms seen in schizophrenia. DA levels were significantly increased in the frontal cortex of group-reared animals receiving MA as well as SIR animals receiving saline, together with a significant reduction is SOD activity. My data suggests that increased DA-mediated oxidative stress may be the driving force in the observed lowering of SOD activity necessary to curb oxidative stress. While no other significant differences were observed in other critical cortico-striatal monoamines (5-HT and NA) among the various treatment and rearing groups, significant differences were observed among monoamine metabolites, including a significant reduction in MHPG in the frontal cortex and striatum of SIR rats receiving MA as well as in the striatum of group-reared rats receiving MA. Moreover DOPAC and HVA were significantly increased in the frontal cortex of group-reared rats receiving MA. The increase in DOPAC and HVA is very likely related to the increased levels of DA present. In conclusion, chronic early postnatal MA abuse during puberty induces profound behavioural and neurochemical deficits closely related to that of schizophrenia, confirming that early life abuse of MA predisposes individuals to developing MA-induced psychosis later in life. However, MA exposure does not necessarily engender a worse outcome in a more susceptible individual, although further studies in this regard are needed.

The current study has also demonstrated that prenatal exposure to MA can partially reproduce some of the behavioural changes akin to schizophrenia. Deficits in social interaction were observed in MA exposed animals, in SIR animals receiving saline as well as SIR animals receiving MA, although no additive effects were observed in the latter. However, no significant differences were observed in PPI, indicating that *in utero* exposure to MA might not induce deficits in sensory motor gating later in life. The lack of effect of SIR alone on PPI was unexpected, but may be related to study design and/or the conditions of study. No differences in cortico-striatal monoamines were observed in any of the treatment and rearing groups,

concurrent with similar-designed studies in the literature. An increase in lipid peroxidation products in the striatum was observed, indicating the presence of oxidative stress. In conclusion, although further study is necessary, my data suggests that MA exposure *in utero* may predispose individuals to develop behavioural abnormalities related to schizophrenia /psychosis later in life, although MA exposure in more susceptible individuals does not necessarily present with a worse outcome.

5.3 Recommendations

Whereas the current dissertation successfully addressed the study objectives defined in Chapter 1, there were a number of limitations to the study, and accordingly a number of recommendations that can be made for future studies.

1. The number of animals employed in the study was in some instances too small, especially in the prenatal group (n=6). However, this was due to an inability to accurately predict the sex of new-born pups prior to birth so that an appropriate number of breeding pairs could be established. This needs to be addressed to enhance the statistical power of the current findings. It would be advisable to budget for more pregnant dams per test group to ensure a larger amount of pups for use in the study. Although a bio-marker of both a cause of oxidative stress as well as a measure of actual oxidative cell damage is required for a study such as this, the small number of animals and limited amount of brain available for analysis after performing the monoamine assay in both brain regions precluded the assessment of both redox parameters. Since only one redox related assay was therefore possible in each of the pre- and postnatal studies, it was decided that SOD activity would be determined in the postnatal group and specifically in the frontal cortex where the most noticeable monoaminergic changes were evident, with lipid peroxidation analyzed in the striatum of prenatally exposed animals. Indeed, lipid peroxidation can originate from sources other than superoxide, making it necessary that a redox parameter such as SOD be undertaken in article 2 (Chapter 4), while analysis in both brain regions is the intended outcome. Despite the limitations, these preliminary findings provide good support that redox dysregulation is evident following pre- and postnatal MA exposure and supports the conclusions of the study.
2. Despite controlling for injections stress in the study design, injection stress may still have significantly affected the study outcomes. Rats were injected twice daily for 16 days (in the postnatal study) while pregnant dams were injected once daily for 16 days (in the prenatal study). Future studies should consider different administration routes, i.e. oral administration via their drinking water.
3. Future studies should investigate the role of glutamate signaling following pre- and postnatal MA administration. In fact some of the behavioural abnormalities as well as

oxidative stress observed in this study may have its origins in increased glutamate and its subsequent effects on monoamine release and redox imbalance. Such an approach may be valuable to our understanding of MA-induced psychosis as well as schizophrenia.

4. In the current study we tested rats on day 78. In addition, testing rats at an age beyond 78 days should be considered in order to determine whether the effects of MA are long-lasting and if they differ markedly from the results obtained in this study.
5. Similarly, behavioural and neurochemical analysis should also be performed immediately following the last dose of MA in order establish a separation between apparent acute MA-induced changes vs. that which occurs later in life.
6. While this study has investigated the effects of MA administration during puberty and *in utero*. It would also be valuable to determine the effects of MA in adulthood and to compare different age groups in order to assess age-related susceptibility to MA induced behavioural or neurochemical abnormalities.
7. In the current study I investigated the effects of MA alone on behaviour and neurochemistry. Future studies should now investigate the effects of an antipsychotic agent administered after MA exposure. This will be valuable in determining effective treatment strategies for patients suffering from MA-induced psychosis.

Addendum A – Materials and Methods

This addendum provides a compilation of all the materials, validations and methods used in this study, including that used to yield results not described in the manuscripts (Chapters 3 and 4), and described in Addendum B. Where these have already been described in Chapters 3 and 4, reference to the appropriate paragraph numbers is provided, with additional detail where appropriate.

A.1 Animals

The study protocol was approved by both the Research Committee and the North-West University (NWU) Ethics committee (ethics approval number NWU – 000105-11-S5), and performed in accordance with the guidelines for the use of animals in experimental work at the North-West University. All animals were maintained according to a code of ethics in research, training and testing of drugs in South Africa and complied with national legislation.

Male and female Wistar rats were supplied by the National Health Laboratory Services, Edenvale. Males weighed approximately 250 g – 300 g on the day of testing. Females were used for breeding and nurturing before weaning, while only male animals were used in the study. Pregnant dams weighed approximately 300 $\pm$ 10 g on the day of giving birth. The rats were randomly assigned to the various treatment groups consisting of between 6 and 10 rats per group.

A.2 Drugs

Methamphetamine hydrochloride (MA) was obtained from Sigma-Aldrich International GmbH (Sigma-Aldrich Manufacturing LLC, 3050 Spruce Street, St Louis MO 63103, United States of America) and diligently documented, stored, dispensed and used as prescribed/allowed by legislation.

The chemical and pharmacological properties of methamphetamine (MA) are discussed in § 2.3.2, § 2.3.3 and § 2.3.4, respectively.

A.2.1 Administration and choice of dosage regimen

Alterations in brain function and development can be observed after MA exposure during early development. A troubling aspect of MA abuse is the current trend of its increased use during adolescence, especially between the ages of 14 and 18 years of age. Exposure to MA during these adolescent years can result in long-term psychiatric and neurological consequences (Kokoshka *et al.,* 2000). In addition, the prevalence of abuse during pregnancy, and hence

prenatal exposure, is also rising (Piper *et al.,* 2011). Also prenatal exposure to MA has been linked to cognitive deficits including attention and visual motor integration impairments later in life. The dopaminergic systems begin to form during prenatal development and continue to change well into adulthood (McFadden *et al.,* 2011). The dopamine transporter (DAT) peaks before adulthood, whereas dopamine (DA) receptors peak during adolescence in both humans and rats (McFadden *et al.,* 2011).

A key feature of stimulant abuse in humans is a gradual escalation of the drug dose before high-dose exposure. To better understand the pathophysiology of MA-induced psychosis, it is important to first look at the pattern of drug abuse. Evidence suggests that neurotoxic effects of MA occur with multiple high-dose administration on a daily basis and these effects can persist long after cessation of MA use. MA abusers usually start with low doses, administered with long intervals between doses, and then progress to higher doses with shorter intervals in between (Segal *et al.,* 2003). In accordance with this known pattern of abuse amongst addicts, the protocol for MA dosing of rats in this study followed a similar course. The purpose of this study was firstly to evaluate whether early life (pre- vs. postnatal) MA exposure can precipitate schizophrenia-like behaviours in rats later in life, and secondly whether the severity of pathology is bolstered in a vulnerable population, in this case in SIR animals, purported to be a neurodevelopmental animal model of schizophrenia.

All treatment regimens in this study involved either prenatal subcutaneous (s.c.) administration to pregnant dams (i.e. prenatal *in utero* exposure of the foetus from prenatal day -13), or postnatal exposure via s.c. administration to rats from postnatal day +35, in both instances for 16 days. Control groups received saline in the same administration regimen. Figure A-1 provides an indication of the group allocation and study outline. In all instances male Wistar rats were used.

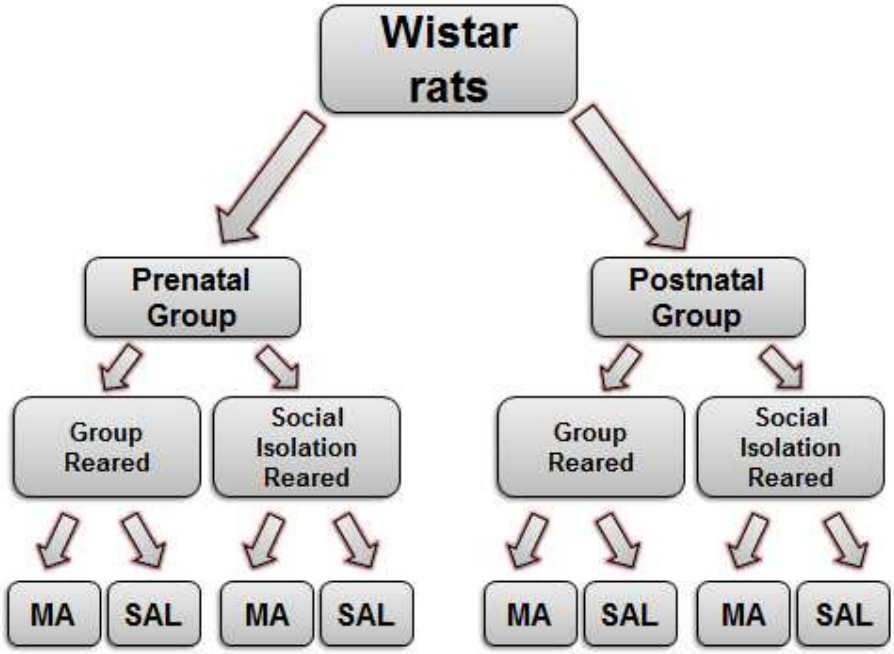

***Figure A-1* – Study layout of prenatal or postnatal Wistar rats group-reared or social isolation reared. SAL – Saline; MA - methamphetamine**

Pregnant dams received 5 mg/kg s.c. MA once daily (Yamamotová *et al.,* 2011; Bubenikova-Valesova *et al.,* 2009; Slamberova *et al.,* 2006) for 16 days, whereas 35 day-old rats received a s.c. escalating dosage regimen as described in Table A-1, for 16 days (Graham *et al.,* 2008; Keller *et al.,* 2011; Kuczenski *et al.,* 2007).

Day	09:00	15:00
1	0.2 mg/kg	0.4 mg/kg
2	0.6 mg/kg	0.8 mg/kg
3	1.0 mg/kg	1.2 mg/kg
4	1.4 mg/kg	1.6 mg/kg
5	1.8 mg/kg	2.0 mg/kg
6	2.2 mg/kg	2.4 mg/kg
7	2.6 mg/kg	2.8 mg/kg
8	3.0 mg/kg	3.2 mg/kg
9	3.4 mg/kg	3.6 mg/kg
10	3.8 mg/kg	4.0 mg/kg
11	4.2 mg/kg	4.4 mg/kg
12	4.6 mg/kg	4.8 mg/kg
13	5.0 mg/kg	5.2 mg/kg
14	5.4 mg/kg	5.6 mg/kg
15	5.8 mg/kg	6.0 mg/kg
16	6.0 mg/kg	6.0 mg/kg

***Table A-1* - Dosage regimen for MA treatment over 16 days from PostND 35 to 50**

A.2.2 General housing and breeding protocol

Rats were housed at the National Health Laboratory Services under conditions of constant temperature (22 ± 2 ℃) and humidity (50%) with a 1 2:12-h light/dark cycle (lights on 06:00 to 18:00). Food and water were provided ad libitum.

For the prenatal treatment group (Fig. A-1) one male Wistar rat was paired with one female Wistar rat for two nights. On the morning of the third day, the male rat was removed and this day was assumed as the day of conception, coined prenatal day -21 (PreND -21). Females were monitored for pregnancy and non-pregnant rats were removed from the study.

The first group of pregnant dams underwent daily treatment, starting on PreND-13 and ending 16 days later (i.e. postnatal day +2 (PostND+2)). Offspring were thus indirectly exposed to MA prenatally via placental transfer for 14 days plus 2 days postnatally via lactation. Half of the group was treated with MA and the other half received daily saline injections (see Fig. A-1). Pups from these dams where later divided into prenatal MA-treated or saline-treated pups, and after weaning on day +21, pups where subdivided into group-reared and social isolation reared (SIR) groups (see § A.2.3). Group-reared rats were housed together in groups of 3-4 rats, where SIR rats were reared in separate cages, 1 rat per cage. Behavioural tests were performed on PostND78.

The second group of pregnant dams underwent no treatment. Pups born from these dams were weaned at PostND21 and divided into group-reared and SIR rats. Treatment started on PostND35 and ended 16 days later (i.e. PostND50), so that these rats were also subdivided into groups receiving MA treatment or saline (see Fig. A-1). Behavioural tests were performed on PostND78.

A.2.3 Social isolation rearing (SIR)

Early-life adverse events like social isolation or maternal separation can affect brain development and behaviour in adulthood, likely contributing to the development of psychiatric disorders such as schizophrenia and depression in genetically predisposed individuals (Fone & Porkess, 2008). Rats are no different from humans in this regard. Post-weaning isolation rearing in rat pups, i.e. preventing social contact with other rats, is associated with an increase in presynaptic DA and 5-HT function in the nucleus accumbens, mesocortical DA hypofunction and altered 5-HT function in the hippocampus and prefrontal cortex (Fone & Porkess, 2008). These changes can result in altered behaviours that strongly resemble core features of schizophrenia, including aggression, cognitive dysfunction, neophobia, deficits in social behaviours and impaired sensorimotor gating. Moreover, MA psychosis might also be more likely to develop or be exacerbated in individuals that have experienced psychosocial or environmental stressors (Grant *et al.,* 2011).

In the isolation rearing procedure, rat pups were housed in individual cages from PostND21 of weaning. Animals were randomly separated into isolation-housed (one animal/cage) and group-reared controls (housed in groups of 3-4 rats/cage), and housed thereafter under controlled ambient conditions (temperature: 21 ± 0.5 ℃, humid ity: 50 ± 10%) and under a continuous 12 h light-dark cycle, with free access to food and water. All groups consisted of 16 rats per group. Solid bottom cages with sawdust were used (Möller, 2009). Isolated rats were not handled more than once a week and had no form of social interaction with other rats, although did have auditory, visual and olfactory contact with rats housed under the same conditions (Fone & Porkess, 2008).

A.3 Background and methods for the behavioural tests

A battery of behavioural tests was carried out in order to evaluate the effect of the different MA treatment regimens on prepulse inhibition (PPI) of startle and social interaction in the presence and absence of isolation rearing. Deficits in PPI and in self- and outward-directed social behaviours in rodents closely resemble well-recognized schizophrenia-like behaviour in humans (Powell *et al.,* 2009) (see § 2.3.8 for further detail). Additional background and method information for each test is provided below.

A.3.1 Prepulse inhibition (PPI)

Studies suggest that schizophrenia patients suffer from pathological changes in the brain resulting in an inability to correctly filter irrelevant sensory stimuli. Thus patients with schizophrenia show a decrease in selective and inhibitory functions of attention that can be measured by paradigms such as prepulse inhibition (PPI) of the startle response (Broberg *et al.,* 2010). In the clinical setting, this paradigm is determined by measuring deficits in eye movement in response to a stimulus. Many patients suffering from schizophrenia exhibit deficits in antisaccade performance, which describes a delay in oculomotor response. In particular it refers to a voluntary eye movement made in the direction opposite to the side where the stimulus is presented. A stimulus is presented to one side and the subject is asked to inhibit a reflex eye movement towards the stimulus. Errors and/or latencies of the antisaccade can indicate dysfunction in the frontal lobe (Ettinger *et al.,* 2005). Schizophrenia patients show a consistent impairment in antisaccade performance (Dyckman *et al.,* 2011)

Prepulse inhibition will be used to provide insight and a better understanding of the similarities in attentional processes between human MA psychosis and the animal model of the same. In rodents an auditory stimulus is used to evoke a startle response. This auditory stimulus is preceded by a prepulse stimulus of a shorter time and lower intensity. A decrease in the startle response after the prepulse is the normal response in a healthy animal, and is referred to as

PPI. Studies have shown deficits in PPI in rats treated with MA and also in patients with schizophrenia (Grant *et al.,* 2011). PPI is a useful index, as it shows similarities with respect to neuropharmacology and neurobiology in both man and rodent. Because of these similarities and also the fact that deficits can be reversed by most antipsychotic drugs, PPI can be useful as a screen for antipsychotic drugs. It is a useful way to measure pre-attentive sensorimotor gating mechanisms that are important for cognitive and sensory information integration. Isolation rearing leads to a definite reduction in PPI of acoustic startle (Fone & Porkess, 2008), and has also recently been replicated in our laboratory (Möller *et al.,* 2011). In this study PPI was used as a behavioural marker to identify schizophrenia-like symptoms in the rat following chronic MA administration similar to that observed in MA psychosis in humans.

PPI was assessed in two sound attenuated startle chambers (SR-LAB, San-Diego instruments, San-Diego, USA), as described in Chapter 3 and 4, § 3.2.3 and § 4.2.3 (see Fig. 2-A). All testing took place between 19:00 – 22:00. The test session consisted of a 5 min acclimatization period with 68 dB white background noise. Four blocks of startle stimuli were then applied, as described in Chapter 3 and 4, § 3.2.3 and § 4.2.3. % PPI was calculated as [(Pulse-alone amplitude – Prepulse + pulse amplitude)/Pulse-alone amplitude] x 100 (Ettinger *et al.,* 2005; Cadenhead, 2011).

Figure A-2: **SR-LAB startle chamber**

A.3.2 Social interaction (SI)

Schizophrenia patients typically show impairments in social functioning, exhibiting a lack of social interaction skills as well as social cognition. Because of this, patients fail to integrate into society (Couture *et al.,* 2006; Möller, 2009).

When a rat is paired with another rat from the same species, certain species-specific behaviours can be observed. Rats will exhibit companion grooming, companion nosing and sniffing and will crawl over or under each other (Grant *et al.,* 2011). Rats treated with MA show

a distinct lack in these behaviours, possibly correlating with social anxiety and paranoid behaviours seen in human MA abusers (Grant *et al.,* 2011). Rats may also exhibit depressive-like symptoms as well as memory deficits (Šlamberová *et al.,* 2010). Social withdrawal can be seen as a negative symptom of schizophrenia (Fone & Porkess, 2008) and alterations in social interaction can therefore be viewed as a useful animal model to study these symptoms (Grant *et al.,* 2011). In a previous study, Möller *et al* (2011) defined various social behaviours related to either positive- (hyperlocomotion, delusions and hallucinations), or negative- (flattened effect, impaired speech and loss of drive) like symptoms following isolation rearing in rats. Since these changes could be reversed by sub-chronic clozapine treatment, this confirms the validity of these tests for schizophrenia. This same array of tests will be studied here.

Numerous methods have been used to discern social behaviour in animals. In one such well-established, validated and commonly used method, 2 unfamiliar animals are placed together and the interaction between the 2 animals is observed (Fig. A-3). In some cases a stressful environment is created and their interaction then observed. Typically SI behaviours decrease as the level of stress is increased (Šlamberová *et al.,* 2010).

Self-directed and social interactive behaviours were assessed in an OFT apparatus (Fig. A-3), described in Chapter 3 and 4, § 3.2.3 and § 4.2.3. Using the OFT test for rats, we determined the extent of indulgence in self-directed behaviours, including squares crossed (locomotor activity) and self-grooming and social interactive behaviours such as rearing, approaching, staying together and anogenital sniffing. Rats were evaluated as a pair for a period of 10 min and the time exhibiting the various behaviours scored in seconds.

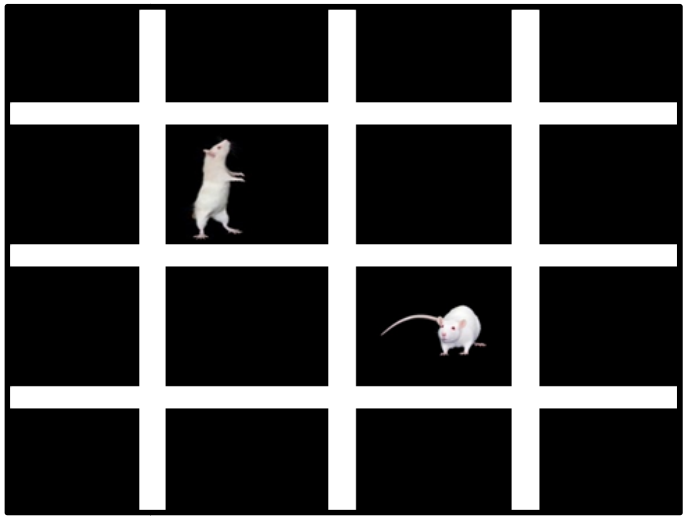

Figure A-3: **OFT apparatus used to assess self-directed and social interactive behaviours. The figure shows the floor divided by white lines in blocks. Opaque black walls of 500 mm in height surround the floor.**

A.4 Neurochemical studies

A.4.1 Preparation of brain tissue

Immediately after the final behavioural tests, rats were sacrificed by decapitation and the striatum and frontal cortex were immediately dissected, as described in § 3.2.4 and § 4.2.4. Immediately afterwards the striatum and frontal cortex were fixed in liquid nitrogen and stored in the -80℃ freezer until the day of assay (§ 3.2.4 and § 4.2.4).

A.4.2 Tissue preparation and regional brain monoamine analysis

Using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), regional quantification of DA, 5-HT and NA, as well as their metabolites, can be detected in the frontal cortex and striatum (as described in § 3.2.4 and § 4.2.4 and Addendum C, § C.1). The concentration of these monoamines will be expressed as ng/mg wet weight of brain tissue (mean ± SEM). The limit of quantification (LOQ) for the monoamine assay is 10 ng/ml. The limit of detection (LOD) is 2.5 ng/ml (Harvey *et al.,* 2006).

A.4.3 Tissue preparation and analysis of lipid peroxidation

Lipid peroxidation was determined using a Lipid Peroxidation (MDA) Assay Kit obtained from *BioVision*®. MDA reacts with TBA, generating a MDA:TBA product that is extracted with butanol and the absorbance read at 532 nm, as described in § 3.2.4 and § 4.2.4 and Addendum C, § C.3.

A.4.4 Tissue preparation and analysis of SOD activity

SOD activity was determined using a commercially available Superoxide Dismutase (SOD) Activity Assay Kit (*BioVision*®). The method for determining SOD activity is described in § 3.2.4 and § 4.2.4 and Addendum C, § C.2.

A.5 Statistical analysis

GraphPad Prism® version 5.00 for Windows (GraphPad Software, San Diego California USA) was used for statistical analysis and graphical presentations. All statistical analyses will be done under the guidance of the Statistical Consultation Service of the North-West University. The unpaired Student's t-test (two-tailed) was performed to compare the data from two treatment groups. Multiple comparisons relative to control were analysed by two-way analyses of variance (ANOVA) followed by Tukey post-test (see §3.3.2.5). Data was presented as averages ± S.E.M. with a value of $p<0.05$ taken as statistically significant.

Addendum B – Additional results

This addendum contains additional data not presented in Chapter 3 (Article 1) or Chapter 4 (Article 2), and acts as supporting material for the main study. For various reasons, but mainly since the addition of these data would have added unnecessary bulk to the articles, this data is presented here. The main study objective of the current study (§ 1.3) was to determine the effects of chronic pre- or postnatal MA administration on late-life behavioural abnormalities, regional brain monoamines and oxidative stress in group-reared and isolation reared male Wistar rats. The materials and methods are fully described in Addendum A and C.

Additional data not found in the articles and that will be presented in this addendum include the following:

- The mean startle amplitude in the PPI test, for the postnatal and prenatal study. This data was analyzed for but not presented in Chapters 3 and 4.
- Monoamine metabolites (MHPG, DOPAC, HVA and 5-HIAA) for the postnatal and prenatal study. This data was analyzed for but not presented in Chapters 3 and 4.

B.1 Postnatal Study

B.1.1 PPI

In the postnatal study (Chapter 3), the PPI test were conducted as described in Addendum A, § A.3.1 and Chapter 3, § 3.2.3. This addendum shows the results for the mean startle amplitudes for the non-treatment and treatment cohort (Fig. B-1-2).

The mean startle amplitudes of the group-reared controls and the isolated rats during four consecutive blocks of ten 120 dB stimuli are shown in Fig. B-1. There were no significant overall differences in mean startle amplitude between the group-reared controls and the SIR animals. There was also no significant habituation observed in the group-reared or SIR animals with repeated stimulation between BLOCK 1 and BLOCK 4 of startle (Fig. B-1).

The mean startle amplitudes of the group-reared control animals receiving saline or MA and the isolated animals receiving saline or MA during four consecutive blocks of ten 120 dB stimuli are shown in Fig. B-2. Again, there were no significant overall differences in mean startle amplitude between the groups. Mean startle amplitude indicates the presence or absence of habituation that may develop when rats are exposed to repeated startling stimuli (Möller *et al*, 2011). The absence of a significant difference as described here indicates that the rats did not develop

habituation. This data adds to the credibility of the results presented in Chapter 3, although is only additive and thus not critically necessary to include in the article.

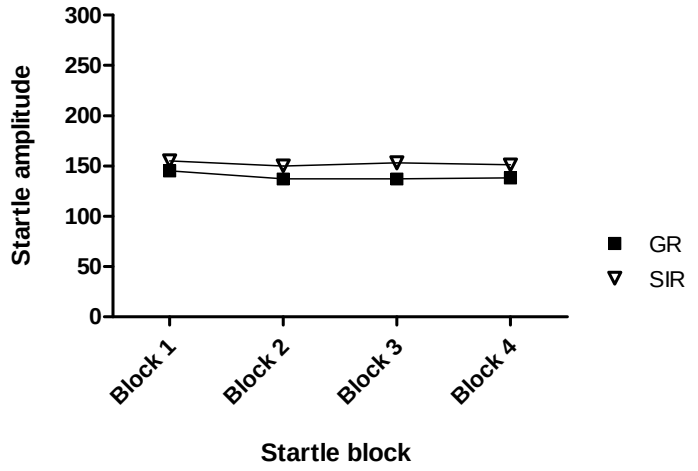

***Figure B-1*: Sensorimotor gating at prepulse intensities as indicated, in group-reared and SIR rats, in the non-treatment cohort (n=10/group) in the postnatal study: mean startle amplitude**

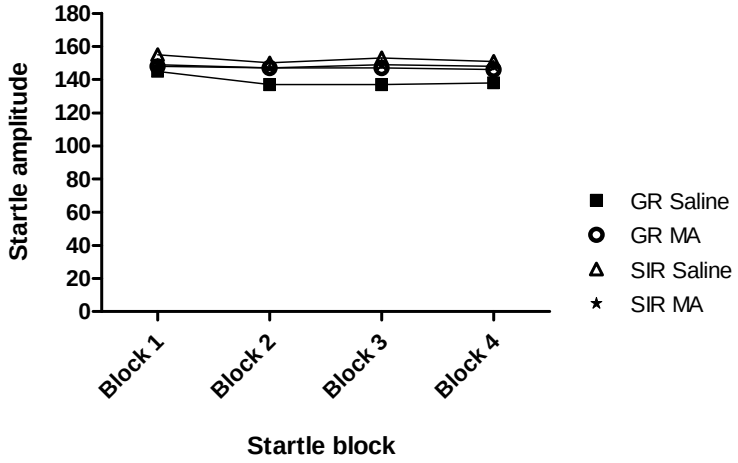

***Figure B-2*: Sensory motor gating at prepulse intensities as indicated, in group-reared and SIR rats in the drug treatment cohort (n=10/group) in the postnatal study: mean startle amplitude**

B.1.2 Metabolites of regional brain monoamines

Along with the monoamines determined in the main study (article 1; NA, DA and 5-HT) the metabolites of these monoamines were also determined, including 3-methoxy-4-hydroxyphenylglycol (MHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 5-hydroxyindole acetic acid (5-HIAA). The methods used

to determine these regional brain monoamines are described in Addendum C and Chapter 3, § 3.2.4.

Two-way ANOVA revealed significant cross-group interactions in the frontal cortex of the drug treatment cohort with respect to MHPG ($F(1,34)=4.344$, $p<0.05$) (Fig. B-3), DOPAC ($F(1,34)=18.828$, $p<0.001$) (Fig. B-4) and HVA) ($F(1,34)=7.760$, $p=0.009$) (Fig. B-5) but no significant interaction for 5-HIAA (Fig. B-6). A significant interaction in MHPG was observed in the striatum ($F(1,33)=5.589$, $p=0.02$) (Fig. B-3). No other significant interactions with respect to monoamine metabolites were observed in the striatum of the drug treatment cohort (Fig. B-4-6).

Post hoc analysis with Tukey revealed a significant decrease in MHPG in the frontal cortex of SIR animals receiving MA ($p<0.001$) when compared to the group-reared control animals receiving saline (Fig. B-3). In the striatum Tukey revealed a significant decrease in MHPG in group-reared animals receiving MA ($p<0.001$) as well as SIR animals receiving MA ($p<0.001$) when compared to their group-reared controls (Fig. B-3).

Post hoc analysis with Tukey revealed a significant increase in DOPAC in the frontal cortex of group-reared animals receiving MA ($p<0.001$) (Fig. B-4) when compared to their group-reared controls. There were no significant differences observed in the striatum (Fig. B-4).

Post hoc analysis with Tukey revealed a significant increase in HVA in the frontal cortex of group-reared animals receiving MA ($p=0.009$) (Fig. B-5) when compared to their group-reared controls. There were no significant differences observed in the striatum (Fig. B-5).

Post hoc analysis with Tukey revealed no significant differences regarding 5-hydroxyindole acetic acid (5-HIAA) in either the frontal cortex or the striatum (Fig. B-6).

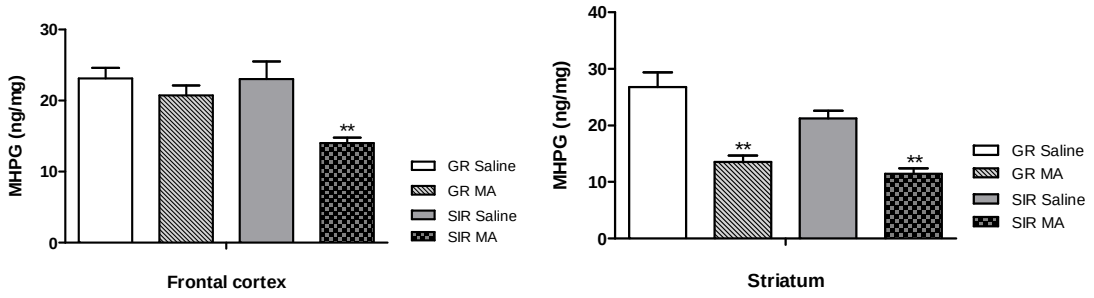

***Figure B-3*: MHPG in the frontal cortex and striatum in group-reared and SIR rats in the treatment cohort (n=10/group) in the postnatal study. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of $p<0.05$ was taken as statistically significant. Statistics: **$p<0.001$ vs. group-reared saline**

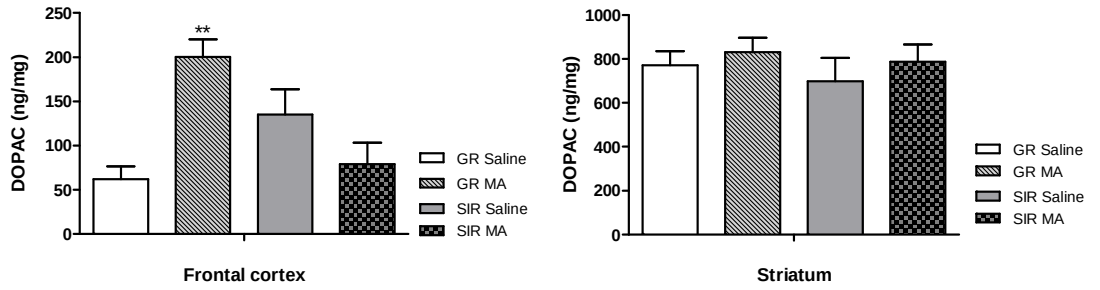

***Figure B-4*: DOPAC in the frontal cortex and striatum in group-reared and SIR rats in the treatment cohort (n=10/group) in the postnatal study. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of p<0.05 was taken as statistically significant. Statistics: **p<0.001 vs. group-reared saline**

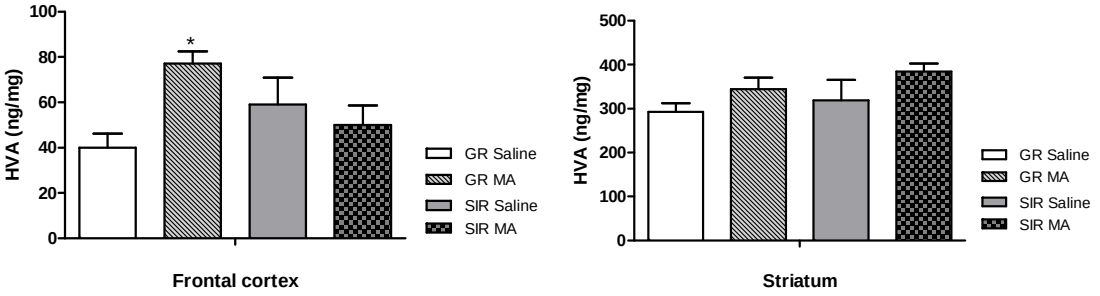

***Figure B-5*: HVA in the frontal cortex and striatum in group-reared and SIR rats in the treatment cohort (n=10/group) in the postnatal study. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of p<0.05 was taken as statistically significant. Statistics: *p<0.01 vs. group-reared saline**

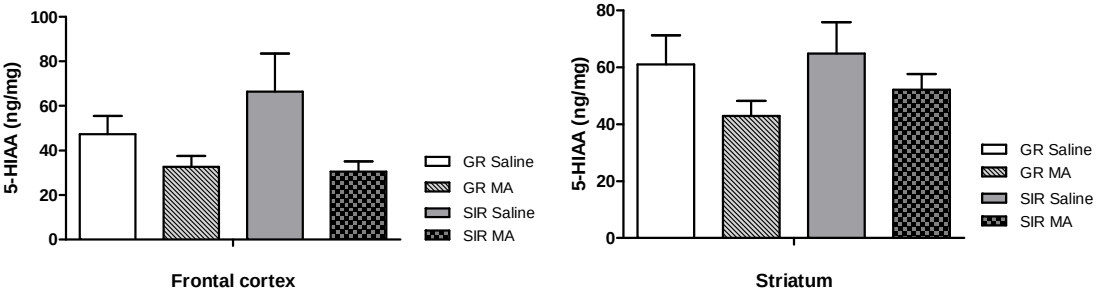

***Figure B-6*: 5-HIAA in the frontal cortex and striatum in group-reared and SIR rats in the treatment cohort (n=10/group) in the postnatal study. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of p < 0.05 was taken as statistically significant.**

In the current study MHPG, a metabolite of NA was reduced in the frontal cortex and striatum of SIR rats receiving MA and reduced in the striatum of group-reared rats receiving MA (Fig. B-3). In both brain regions, no significant differences were observed in SIR rats receiving saline. NA is metabolized via monoamine oxidase (MAO) to 3,4-dihydroxyphenylglycol (DHPG) which in

turn is metabolized via catechol-*O*-methyltransferase (COMT) to produce MHPG (Eisenhofer *et al.,* 2004). MA administration inhibits MAO (Cruickshank & Dyer, 2009) resulting in the decreased levels of MHPG observed here, even though NA levels (article 1, Chapter 3) in the frontal cortex and striatum of all the groups were not significantly different.

An increase in DOPAC in the frontal cortex was observed in group-reared animals receiving MA (Fig. B-4), with a slight but non-significant elevation in SIR animals receiving saline. However, no differences in DOPAC were observed in the striatum. DA is metabolized via MAO into DOPAC and DOPAC is further metabolised via COMT to HVA (Quaak *et al.,* 2009). This increase in DOPAC in grouped-reared animals receiving MA is inconsistent with previous studies stating that MA inhibits MAO (Cruickshank & Dyer, 2009), in which case the metabolism of DA to DOPAC should be inhibited with an eventual reduction in DOPAC as presented here, as seen in SIR rats receiving MA (Fig. B-4). The increase in DOPAC can be explained by the increase in DA observed in the frontal cortex of group-reared rats receiving MA (article 1, Chapter 3), where more DA means more DOPAC produced after oxidative metabolism. In SIR rats receiving saline where no MA is present to inhibit MAO, the aforementioned increase in DA translates into higher levels of DOPAC. By the same argument, in the striatum where there is no significant difference in DOPAC between the rats receiving MA and their group-reared controls (Fig. B-4), this could possibly be as a result of decreased DA concentrations in the striatum (see article 1, Chapter 3).

The increase in HVA in the frontal cortex of group-reared rats receiving MA (Fig. B-5) can be attributed to the increased DA (see article 1, Chapter 3) that is available for metabolism to HVA (Quaak *et al.,* 2009). There was no significant difference in striatal HVA observed between the groups because there was no significant difference in striatal DA concentrations.

No differences were observed in 5-HIAA in either the frontal cortex or striatum (Fig. B-6). 5-HT is metabolized via MAO to 5-HIAA (Squires *et al.,* 2010). The acute administration of MA results in the release of 5-HT (Yamamoto *et al.,* 2010). Chronic administration causes long-term depletion of monoamines such as 5-HT and its metabolites (Imam & Ali, 2001; Grace *et al.,* 2010), which may explain these findings. Indeed, we did not find any change in 5-HT in any of the treatment groups (see article 1, Chapter 3).

While the reporting of monoamine metabolites is of value, they did not add immediate value to the interpretation of my findings in article 1 (Chapter 3). They also added unnecessary bulk to the manuscript, and for these reasons it was decided to present these data here.

B.2 Prenatal Study

B.2.1 PPI

In the prenatal study (Chapter 4), the PPI test were conducted as described in Addendum A, § A.3.1 and Chapter 4, § 4.2.3. This addendum shows the results for the mean startle amplitudes for the non-treatment and treatment cohort (Fig B-7-8).

The mean startle amplitudes of the group-reared controls and the isolated rats during four consecutive blocks of ten 120 dB stimuli are shown in Fig. B-7. There were no significant overall differences in mean startle amplitude between group-reared controls and SIR animals. There was also no significant habituation observed in the group-reared or SIR animals with repeated stimulation between BLOCK 1 and BLOCK 4 of startle (Fig. B-7).

The mean startle amplitudes of group-reared control animals receiving saline or MA and the isolated animals receiving saline or MA during four consecutive blocks of ten 120 dB stimuli are shown in Fig. B-8. There were no significant overall differences in mean startle amplitude between the groups. As mentioned in § B.1.1. mean startle amplitude indicates if habituation develops with repeated startling stimuli (Möller *et al*, 2011). No significant differences indicated that rats did not develop habituation. This data adds to the overall credibility of the results presented in chapter 4 (article 2), although is only additive and not critically necessary to include in the manuscript.

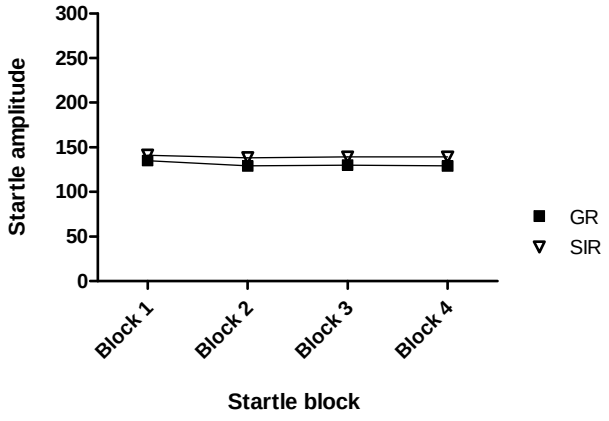

***Figure B-7*: Sensorimotor gating at prepulse intensities as indicated, in group-reared and SIR rats, in the non-treatment cohort (n=6/group) in the prenatal study: mean startle amplitude**

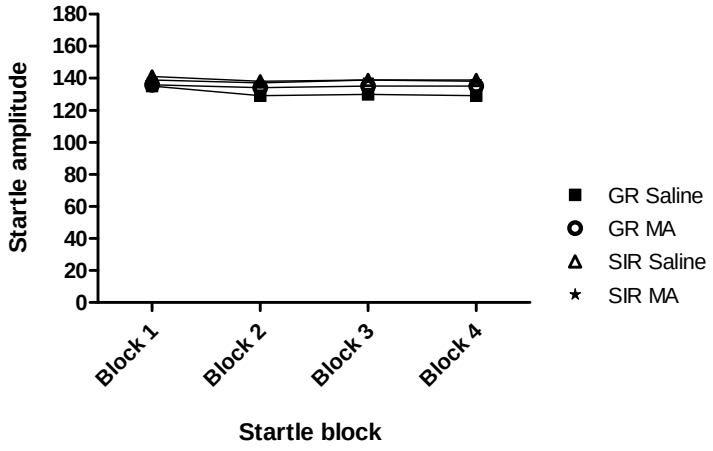

***Figure B-8*: Sensory motor gating at prepulse intensities as indicated, in group-reared and SIR rats in the drug treatment cohort (n=6/group) in the prenatal study: mean startle amplitude**

B.2.2 Metabolites of regional brain monoamines

Along with the monoamines determined in the main study (article 2, Chapter 4; NA, DA and 5-HT) the metabolites of these monoamines were also determined, and are described here. The methods used to determine these regional brain monoamines are described in Addendum C and Chapter 4, § 4.2.4.

Two-way ANOVA revealed no significant cross-group interactions in the frontal cortex or striatum of the drug treatment cohort with respect to MHPG ($F(1,19)=5.107$, $p=0.16$, frontal cortex and $F(1,19)=1.234$, $p=0.281$, striatum), DOPAC ($F(1,19)=0.282$, $p=0.601$, frontal cortex and $F(1,19)=0.003$, $p=0.956$, striatum), HVA ($F(1,19)=0.923$, $p=0.349$, frontal cortex and $F(1,19)=1.634$, $p=0.217$, striatum) and 5-HIAA ($F(1,19)=0.272$, $p=0.608$, frontal cortex and $F(1,19)=1.812$, $p=0.194$, striatum) (Fig. B-9-12).

Post hoc analysis with Tukey revealed no significant differences regarding MHPG in either the frontal cortex or striatum (Fig. B-9).

Post hoc analysis with Tukey revealed no significant differences regarding DOPAC in either the frontal cortex or striatum (Fig. B-10).

Post hoc analysis with Tukey revealed no significant differences regarding HVA in either the frontal cortex or the striatum (Fig. B-11).

Post hoc analysis with Tukey revealed no significant differences regarding 5-HIAA in either the frontal cortex or the striatum (Fig. B-12).

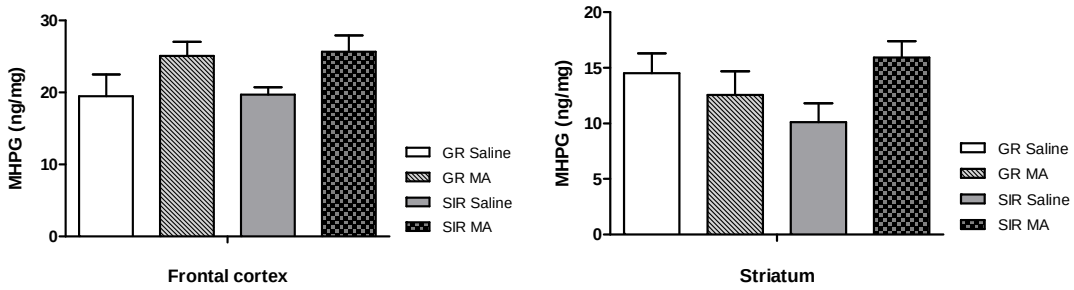

***Figure B-9*: MHPG in the frontal cortex and striatum in group-reared and SIR rats in the treatment cohort (n=6/group) in the prenatal study. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of p<0.05 was taken as statistically significant.**

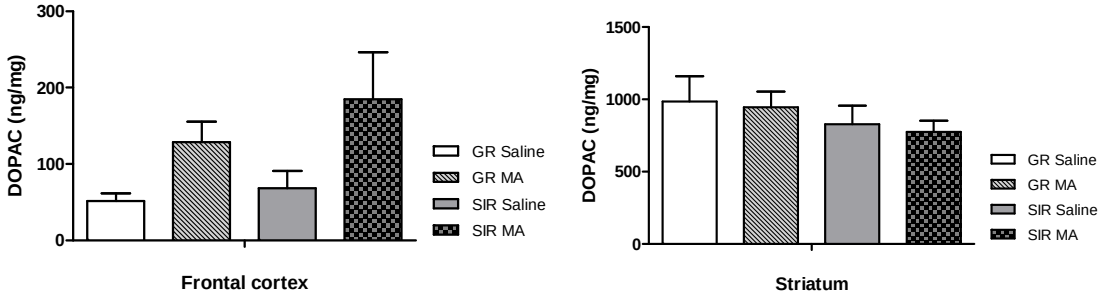

***Figure B-10*: DOPAC in the frontal cortex and striatum in group-reared and SIR rats in the treatment cohort (n=6/group) in the prenatal study. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of p<0.05 was taken as statistically significant.**

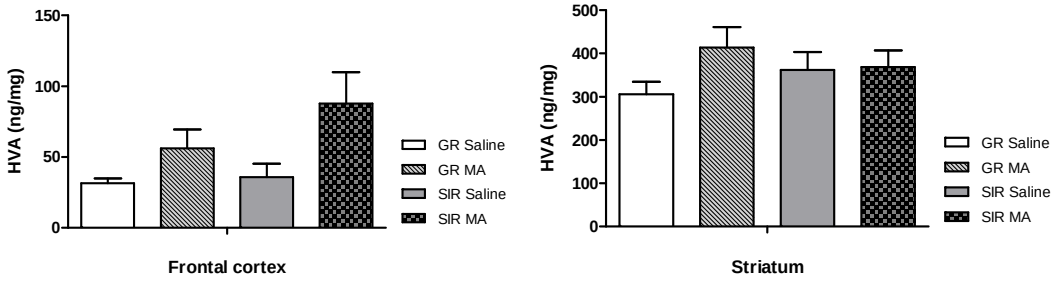

***Figure B-11*: HVA in the frontal cortex and striatum in group-reared and SIR rats in the treatment cohort (n=6/group) in the prenatal study. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of p<0.05 was taken as statistically significant.**

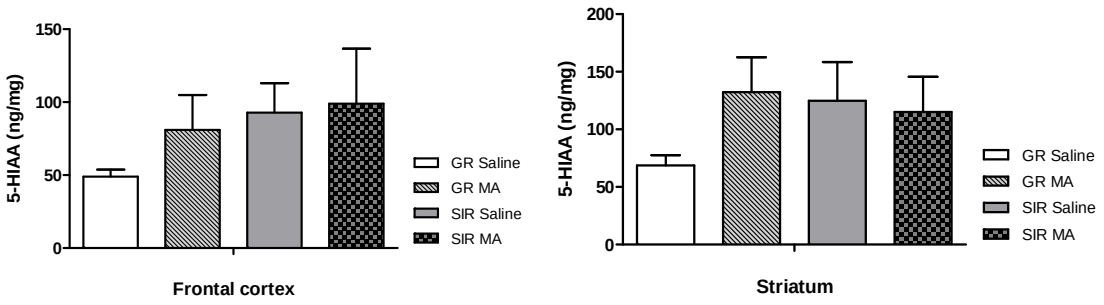

***Figure B-12*: 5-HIAA in the frontal cortex and striatum in group-reared and SIR rats in the treatment cohort (n=6/group) in the prenatal study. A two-way ANOVA followed by a Tukey posttest was performed to compare the different drug treatment groups with their respective control. A value of $p < 0.05$ was taken as statistically significant.**

Studies have shown that although monoamine levels are elevated in rats during prenatal exposure, adult offspring show normal levels of DA and 5-HT (Heller *et al.,* 2001a). In the current study I observed no significant differences in brain monoamines (NA, DA and 5-HT; article 2, Chapter 4), supporting this. Because there was no significant difference in monoamines, it may be projected that changes in their metabolites is unlikely.

While the reporting of monoamine metabolites is of value, they did not add immediate value to the interpretation of my findings in article 2. They also added unnecessary bulk to the manuscript, and for these reasons it was decided to present these data here.

The complete summary and discussion of all the results in Chapter 3, Chapter 4 and Addendum B can be found in Chapter 5.

Addendum C – Neurochemical analysis

The following addendum includes valuable and additional information to that described in articles 1 and 2 concerning the methodology and experimental procedures in the preparation of the brain tissue and measurement of brain monoamines, superoxide dismutase (SOD) activity and lipid peroxidation.

C.1. Regional brain monoamine analysis

C.1.1 Introduction

Following the final behavioural tests, rats were sacrificed by decapitation and the striatum and frontal cortex was immediately dissected, as described in § 3.2.4 and § 4.2.4. Following the brain dissection, the frontal cortex and striatum of each animal was placed individually into polypropylene tubes, marked and frozen with liquid nitrogen. Frozen brain tissue collected after sacrifice were then used for regional quantification of DA, 5-HT and NA, as well as their metabolites, using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (Harvey *et al.,* 2006, Harvey *et al.,* 2010). Monoamine concentrations in the samples will be determined by comparing the area under the peak of each monoamine in the sample to that of the monoamine standard, isoprenaline (range 5–50 ng/ml; Chemstation Rev. A 06.02 data acquisition and analysis software). Linear standard curves (regression coefficient greater than 0.99) are found in this range. Monoamine concentrations will be expressed as ng/mg wet weight of tissue (mean ± SEM).

C.1.2 Materials and methods

C.1.2.1 Chromatographic conditions

Analytical Instrument: Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem III Electrochemical detector (with Coulometric flow cell) and Chromeleon® Chromatography Management System version 6.8.

Column: Kinetix C18 column, 150 x 4.6 mm, 2.6µm, 100 Å pores, (Phenomenex, Torrance, CA).

Mobile Phase: 0.1 M Sodium formate buffer, 0.5 mM ethylene-diaminetetraacetic acid (EDTA dinatriumsalt Na_2EDTA), 5 mM sodium heptane

sulphonic acid, 5% v/v acetonitrile. The pH of the mobile phase was set at ± pH 4.10 with orthophosphoric acid (10%).

NOTE: ***Method is highly pH sensitive.***

Flow rate: 0.30 ml/min.

Injection volume: 10 µl

ECD Detector settings: ESA 5011A Analytical Cell Potential settings,

Volts: E1: +150mV (or +0.150V)

E2: +600mV (or +0.600V)

Gainrange: 20nA,

Polarity: Positive,

Reaction: Oxidation,

Guardcell Potential setting: +50mV

C.1.2.2 Chemicals and reagents

Solution A

Contents of Solution A:

0.5 mM sodium metabisulphite

0.3 mM Na_2EDTA

0.1 M perchloric acid (60% strong solution).

Preparation of Solution A:

1. Weigh off 0.09505 g sodium metabisulphite and 0.111672 g Na_2EDTA and dissolve in 800 ml distilled water.
2. Add 10.87 ml perchloric acid to above solution and make up to 1000 ml.

Note: All standards will be prepared with this solution.

C.1.3 Sample preparation of brain tissue samples

1. Following brain dissection, the tissue of each animal was placed individually into polypropylene tubes, marked and frozen with liquid nitrogen.
2. On the day of analysis, sample were weighed, thawed and 1 ml of solution A was added to each tube. The tissue in each tube was then ruptured by sonication (2 x 12 seconds, at an amplitude of 14 µ; Fig. C-1A).

3. The tubes were allowed to stand on ice for 20 minutes to complete perchlorate precipitation of protein and extraction of monoamines.
4. Following this period, samples were centrifuged at 4ºC in an ultra-centrifuge for 20 minutes at 16 000 rpm (24 000 g; Fig. C-1B).
5. The pH of the sample was adjusted to pH5 with the addition of 1 drop/ml of 10 M potassium acetate.
6. An aliquot of 200 µl of the tissue extract was removed with a pipette and placed into another tube.
7. 20 µl of the internal standard, isoprenaline, was added to the sample.
8. The final sample was vortexed (Fig. C-1C), centrifuged and whereupon 10 µl was injected onto the HPLC column.
9. The results were expressed as ng/mg wet weight of tissue.
10. The limit of quantification (LOQ) for the monoamine assay is 10 ng/ml. The limit of detection (LOD) is 2.5 ng/ml (Harvey *et al.,* 2006).

***Figure C-1: (*A) Sonicater, (B) Centrifuge and (C) Vortex**

C.2. Analysis of superoxide dismutase (SOD) activity

C.2.1 Introduction

One of the most important antioxidant enzymes of the body is SOD, which catalyzes the dismutation of the superoxide anion (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), thereby providing an important defence against oxidative damage. The sensitive SOD Activity Assay Kit® (BioVision) utilizes a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt), that produces a water-soluble formazan dye upon reduction with superoxide anion (O_2^- ; Fig. C-2). The rate of the reduction of WST-1 with O_2^- is linearly related to the activity of xanthine oxidase

(XO). This reduction process is inhibited by SOD with the inhibitory action measurable by a colorimetric method.

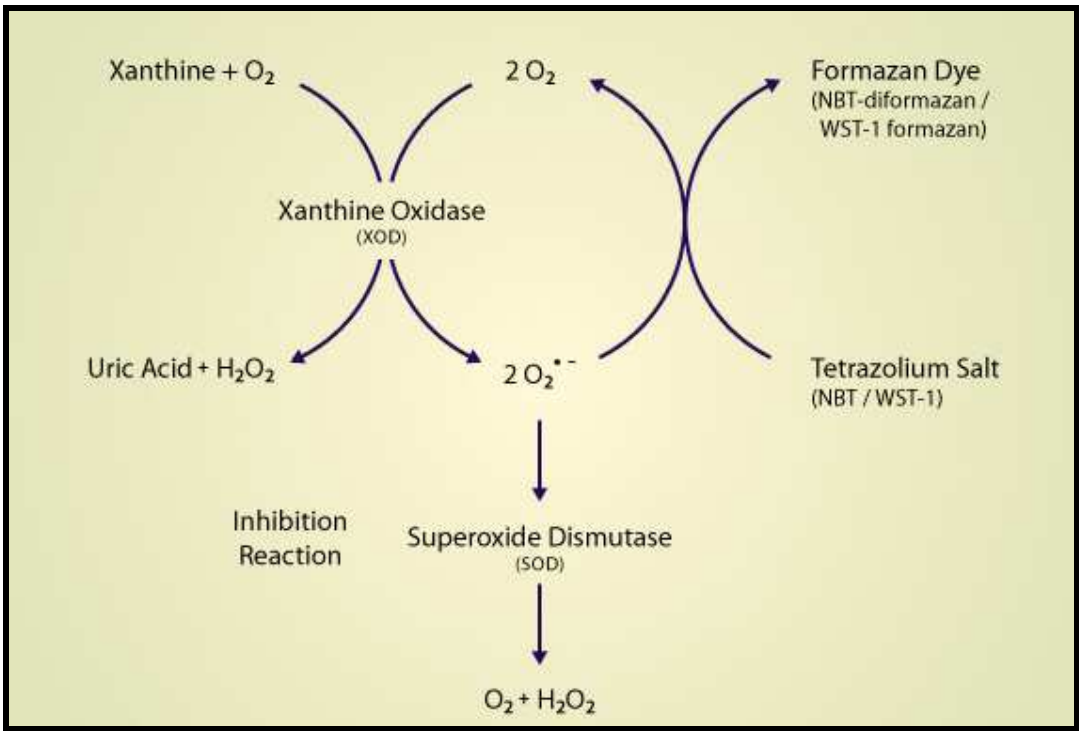

Figure C-2: **Principle of the SOD Activity Assay Kit (BioVision®)**

C.2.2 Materials and methods

C.2.2.1 Chemicals and reagents

All chemicals and reagents used in this study were of the highest grade, purchased from BioVision®, Johannesburg, South Africa.

Kit components:

- WST Solution
- SOD Enzyme Solution
- SOD Assay Buffer
- SOD Dilution Buffer

C.2.2.2 Preparation of working solutions for the SOD activity assay

- ***WST working solution:*** Dilute 1 ml of WST solution with 19 ml of Assay Buffer Solution
- ***Enzyme working solution:*** Vortex the Enzyme solution tube for 5 seconds. Mix well by pipetting. Dilute 15 µl with 2.5 ml of Dilution Buffer.

C.2.2.3 Preparation of brain homogenate

- ***Preparation of phosphate buffered solution (PBS):*** For 2.5 liters of a 100x strength solution, add 200 g NaCl, 5 g KCl and 22.5 g Na_2HPO_4 to double distilled water and make up to 2.5 liters. On the day of assay, 1 part PBS was diluted with 9 parts double distilled water (ddH_2O) before use to obtain a 0.01 mM PBS dilution.

Rats were sacrificed by decapitation as described in § C.1.1. On the day of the SOD assay, the brain regions needed for analysis (frontal cortex) was removed from the -80℃ freezer, allowed to thaw on ice and weighed. A 10% w/v solution was then made with the brain samples in ice-cold PBS, pH 7.4.

Thereafter, tissue samples were sonicated and the resulting homogenates centrifuged at 14000 xg for 10 minutes at 4℃. The supernatant contains total SOD activity and was used for the SOD activity assay and the determination of protein content.

C.2.2.4 Protein determination

Protein determination of the brain homogenate was carried out according to the method of Bradford (Bradford, 1976). The Bradford reagent was shaken lightly and an adequate amount was removed and kept at room temperature. Protein standards were prepared by dissolving 2 mg bovine serum albumin (BSA) in 1 ml ddH_2O, producing a 2 mg/ml solution. A series of 100 µl dilutions were then made, as indicated in Table C-1.

	Dilution in test tubes	
Protein concentration	**Volume of 2 mg/ml BSA**	**Volume of PBS buffer**
0 mg/ml (blank)	0 µl	100 µl
0.1 mg/ml	5 µl	95 µl
0.4 mg/ml	20 µl	80 µl
0.7 mg/ml	35 µl	65 µl
1.0 mg/ml	50 µl	50 µl
1.4 mg/ml	70 µl	30 µl

Table C-1: **Protein concentration standards**

5 µl of each tube (from the blank, standards and brain homogenate) was added to separate wells of a 96-well plate in duplicate. Thereafter, 250 µl of the Bradford reagent was added to each well using a repeat pipettor or micropipette and immediately mixed on a shaker (e.g. the shaking facility of the plate reader) for ±30 seconds. The plate was incubated at room temperature for ±15 minutes (5 – 45 min, max 60 min) and the absorbance determined in a plate reader (multiscan plate reader, AEC Amersham) using the 560 nm filter. The average blank absorbance value was then subtracted from all other absorbance values (net absorbance

values) and “net absorbance vs. protein concentration of the standards” plotted to determine the unknown protein concentration.

C.2.2.5 Determination of SOD activity in brain homogenate

Refer to Table C-2 for the amount of solution in each well. Refer to Figure C-3 for layout of 96-well plate.

- Add 20 µl of the 10% brain homogenate (Sample solution) to each sample and blank 2 well and add 20 µl H_2O to each blank 1 and blank 3 well. Each sample of the brain homogenate was assayed in duplicate.
- Add 200 µl of the WST Working Solution to each well.
- Add 20 µl of the Dilution Buffer to each blank 2 and blank 3 well.
- Add 20 µl of the Enzyme Working Solution to each sample and blank 1 well using a multiple channel pipette to avoid reaction time lag of each well. Mix thoroughly.
- Incubate the plates (Fig. C-3) at 37℃ for 20 minutes.

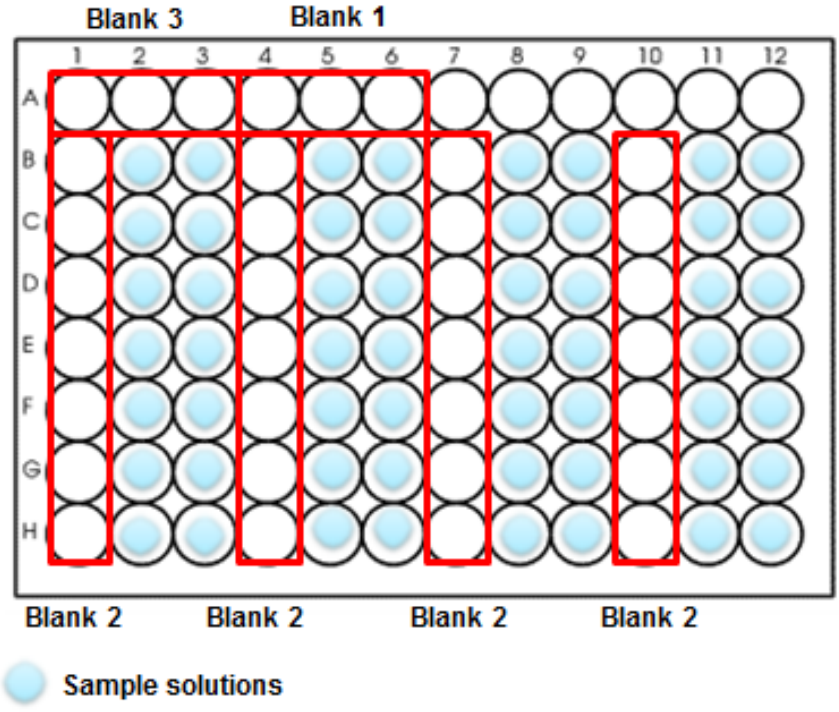

Figure C-3: **Layout of 96-well plates for the SOD assay**

- Read the absorbance at 450 nm using a microplate reader
- The % SOD activity (inhibition rate %) was calculated using the following equation:

$$\underline{(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})}$$

SOD Activity (inhibition rate %) = $(A_{blank1} - A_{blank3})$ **x100**

- For tissue samples, as used in the current study, one unit (U) of enzyme activity is defined as the quantity of SOD required to cause a 50% inhibition of the absorbance change per minute of the blank reaction (diluent rate). Units of SOD activity in the frontal cortex and striatum are expressed as U/mg protein.

	Sample (10% brain homogenate)	**Blank 1**	**Blank 2**	**Blank 3**
Sample solution	20 µl		20 µl	
ddH_2O		20 µl		20 µl
WST Working solution	200 µl	200 µl	200 µl	200 µl
Enzyme Working solution	20 µl	20 µl		
Dilution Buffer			20 µl	20 µl

Table C-2: **Amount of each solution for sample, blank1, 2 and 3**

C.3. Analysis of lipid peroxidation

C.3.1 Introduction

Quantification of lipid peroxidation is necessary to assess oxidative stress-mediated cell damage in pathophysiological processes. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are natural end products formed by lipid peroxidation (Fig. C-4). Measuring these products is one of the most widely accepted assays for the determination of oxidative stress (Hall & Bosken, 2009). The Lipid Peroxidation Assay Kit® (BioVision) is used for sensitive detection of MDA in a variety of samples. MDA in samples is reacted with thiobarbituric acid (TBA) to generate the MDA-TBA adduct (Fig. C-4) that can be quantified colorimetrically (at a wavelength of 532 nm) using a multiscan plate reader (AEC Amersham). The assay detects MDA levels as low as 1 nm/well colorimetrically.

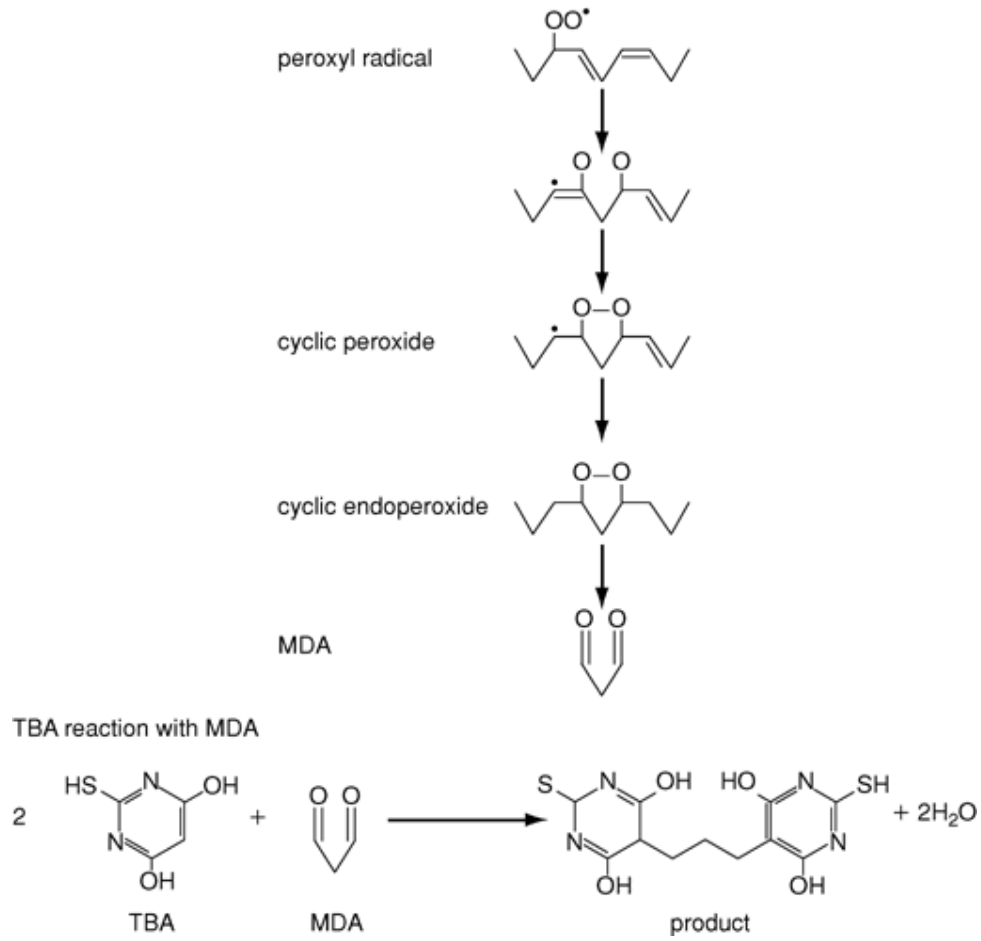

Figure C-4: **Formation of MDA and the MDA-TBA adduct (Hall & Bosken, 2009)**

C.3.2 Materials and methods

C.3.2.1 Chemicals and reagents

All chemicals and reagents used in this study were of the highest grade, purchased from BioVision®, Johannesburg, South Africa. Briefly vortex all vials before opening. Allow all components to warm to room temperature before use.

Kit components:

- MDA Lysis Buffer
- BHT (100x)
- TBA
- MDA Standard (4.17 M)

C.3.2.2 Preparation of solutions

- ***TBA Solution:*** Reconstitute one vial of TBA with 7.5 ml glacial acetic acid and adjust the final volume to 25 ml with ddH_2O. Use within 6-8 hours.

- ***MDA Standard Curve:*** Dilute 10 µl of the MDA standard with 407 µl ddH_2O to prepare a 0.1 M MDA solution. Dilute 20 µl of the 0.1 M MDA solution with 980 µl ddH_2O to prepare a 2 mM MDA Standard. For colorimetric analysis, add 0, 2, 4, 6, 8 and 10 µl of the 2 mM MDA Standard into separate microcentrifuge tubes and adjust the final volume to 200 µl with ddH_2O to generate 0, 4, 8, 12, 16 and 20 nmol Standard per well.

C.3.2.3 Preparation of brain homogenate

Rats were sacrificed by decapitation as described in § C.1.1. On the day of the lipid peroxidation assay, the brain regions needed for analysis (striatum) was removed from the -80℃ freezer, allowed to thaw on ice and weighed. 3 00 µl of the MDA Lysis Buffer (with 3 µl BHT) was added to 10 mg tissue and then sonicated. The resulting homogenate was then centrifuged at 13000 xg for 10 minutes to remove insoluble material. 200 µl of the supernatant from each sample was placed in a microcentrifuge tube.

C.3.2.4 Protein determination

Protein determination of the brain homogenate was carried out according to the method of Bradford (see §C.2.2.4).

C.3.2.5 Determination of MDA in brain homogenate

- Add 600 µl of TBA solution into each vial containing standard and sample
- Incubate at 95℃ for 60 minutes
- Cool to room temperature in an ice bath for 10 minutes
- Centrifuge at 16000 xg for 3 minutes
- Pipette 200 µl from each 800 µl reaction mixture into a 96-well microplate for analysis (Fig. C-5). Each sample was assayed in duplicate.

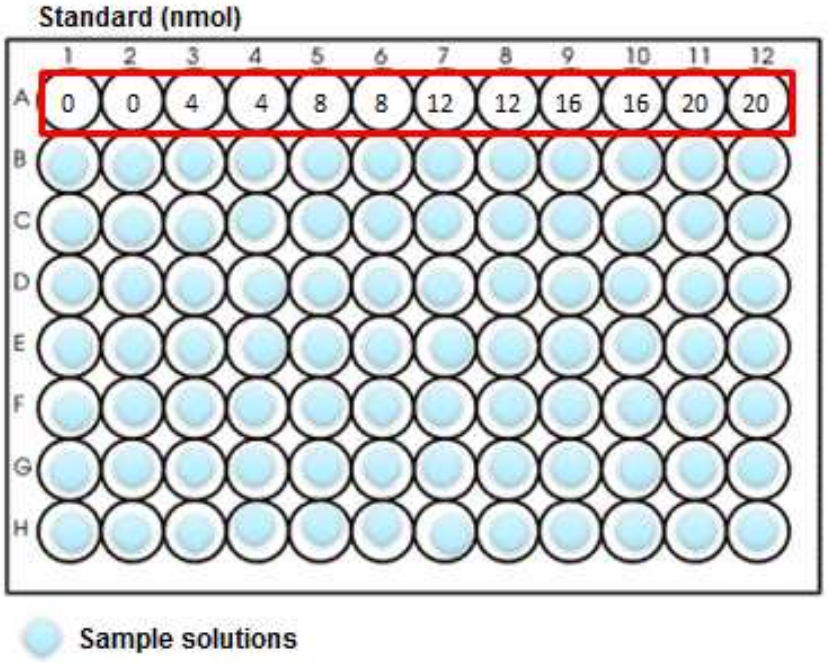

Figure C-5: **Layout of the 96-well plate for the lipid peroxidation assay**

- Read the absorbance at 532 nm (colorimetric analysis)
- Plot the MDA Standard Curve (Fig. C-6), then calculate the MDA amount in the test sample using the equation below.
- Determine the sample amount of MDA equivalents in nmol by interpolation from the standard curve. Correct sample values for any other dilutions performed during specimen preparations.

C = (A/mg) x 4 x D = nmol/mg

- **C = concentration in nmol/mg**
- **A : Sample MDA amount from the standard curve (in nmol)**
- **mg : Original tissue amount used (e.g. 10 mg)**
- **4 : Correction for using 200 µl of the 800 µl reaction mix**
- **D : Dilution factor (if any BEFORE original amount or volume)**

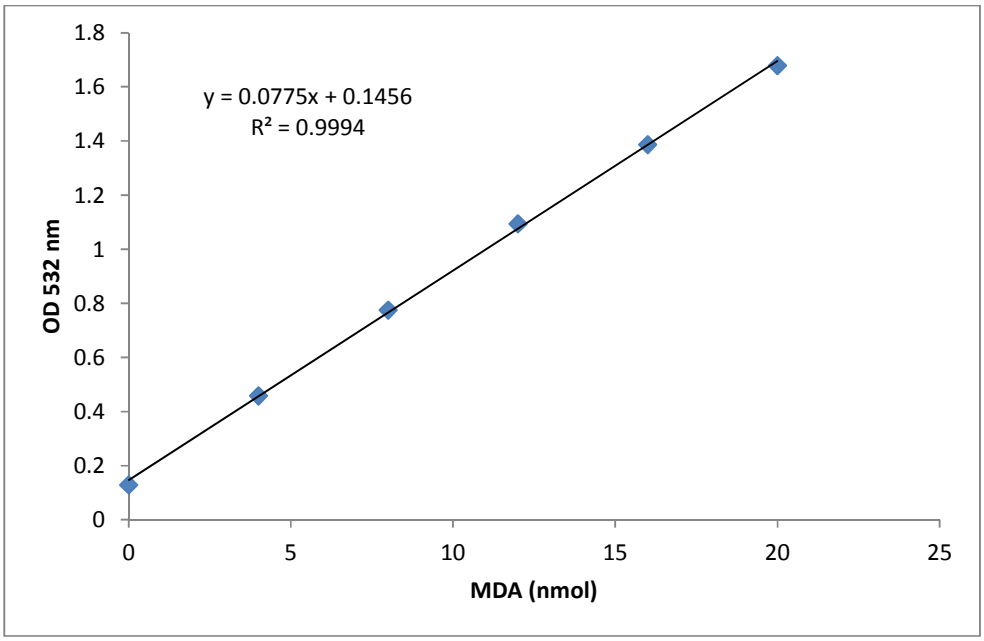

Figure C-6: **MDA Standard curve, with a R^2 of 0.9994**

Addendum D - Instructions to the author

The inclusion of the “Instructions to the authors” is to indicate the guidelines given by the specific journals to which the articles in Chapter 3 and Chapter 4 will be submitted to.

The Instructions to the authors pertaining to **Developmental Neuroscience** can be found at:

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Developmental neuroscience

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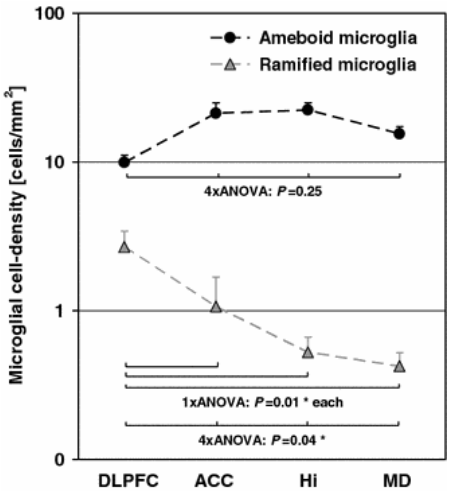

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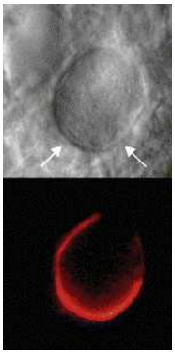

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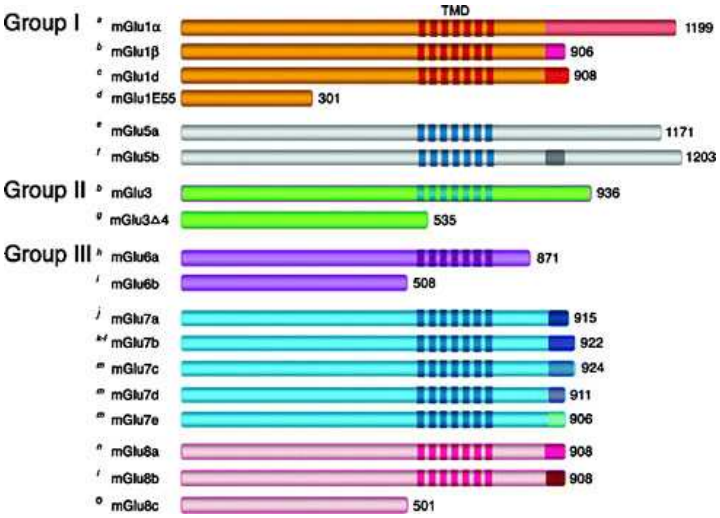

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Addendum E - Congress contribution

In this addendum the study abstract that was presented at the congress of the South African Basic and Clinical Pharmacology Society (2012, Pretoria, South Africa) Young Scientist Category, is shown.

Effects of chronic methamphetamine exposure during puberty on late-life social interactive and sensorimotor gating behaviours in social and isolation reared rats

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Purpose

Globally methamphetamine (MA) is the second most widely abused drug after cannabis. Much of this phenomenon is due to its low manufacturing cost and accessibility. In South Africa MA is commonly referred to as "TIK" and its abuse is especially a problem in the Western Cape. MA abuse predominantly occurs between early adolescence and adulthood (<30 years). Abuse during this period of development introduces the young adult to a high risk of neuro-developmental insults that can affect neuronal maturity and function with implications for neuro- and psychopathology later in life. Importantly chronic MA abuse results in a psychosis indistinguishable from paranoid schizophrenia. Schizophrenia also underlies an early life neuro-developmental abnormality brought about by adversity. Consequently chronic MA abuse may sensitize certain at-risk individuals to developing schizophrenia later in life. Using social isolation rearing (SIR), a neuro-developmental animal model of schizophrenia, we investigated the ability of chronic MA administration to modify SIR-induced behavioural changes akin to schizophrenia. We focussed specifically on the post-natal pubertal period equating to adolescence/early adulthood in man.

Methods

Pups born of MA-naive female Wistar dams were divided into SIR and group-reared animals immediately post-weaning (on postnatal day +21). Young rats subsequently received either saline (2ml/kg s.c. bid) or an escalating dose of MA (0.2 mg/kg s.c. bid – 6 mg/kg s.c. bid) for 16 days from postnatal day +35 until postnatal day +50 (puberty to early adulthood). On postnatal day +78, male rats (250-300g; n=10/group) were tested for deficits in social interactive behaviours and prepulse inhibition (PPI). The rats were sacrificed after testing. The study was approved by the Research Ethics Committee of North-West University (NWU-000105-11-S5).

Results

SIR significantly reduced outward-directed social behaviours (rearing time, approaching and together time, anogenital sniffing) but increased self-directed behaviour (self-grooming), behaviours that are closely related to the social deficits evident in schizophrenia. SIR also induced profound deficits in PPI, as is noted in schizophrenia. Pubertal MA exposure in group-reared and SIR animals induced similar deficits in PPI and in most instances was greater than that in saline treated group-reared animals. Pubertal MA exposure in group-reared animals similarly affected all outward-and self-directed social behaviours (viz. approaching and together time, anogenital sniffing, grooming), except time rearing and squares crossed. In many criteria these changes were similar in extent to that in SIR animals, and greater than saline control. Squares crossed, a measure of locomotor activity, was only elevated in SIR-saline and MA plus SIR animals vs. saline group-reared controls. The data thus demonstrates that MA and SIR separately are similarly effective in evoking schizophrenia-like behaviours in rodents, thus confirming the psychogenic nature of chronic early life MA exposure. Chronic MA exposure in a high risk population, i.e. MA plus SIR, did not demonstrate a greater penchant for developing more severe psychopathology, although reduction in times approaching and anogenital sniffing (measures of outward-directed behaviours) do show a more robust response in SIR plus MA exposed animals.

Addendum F - Abbreviations

Numerals

3 – OHAA	3-Hydroxyanthranilic acid
4 - HNE	4-Hydroxynonenal
5 – HIAA	5-Hydroxyindole acetic acid
5 – HT	5-Hydroxytryptophan (Serotonin)
5 – HT *x*	5-Hydroxytryptophan (Serotonin) *x* – receptor subtype

A

ADHD	Attention-deficit/hyperactivity disorder
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-5-hydroxy-3-methyl-4-isoxazde propionic acid
AP-1	Activating protein-1
ATP	Adenosine triphosphate

B

BBB	Blood-brain barrier

C

Ca^{2+}	Calcium
COMT	Catechol-*O*-methyltransferase
CNS	Central nervous system
CSF	Cerebrospinal fluid

D

D *x*	Dopamine *x*-receptor subtype
DA	Dopamine
DAT	Dopamine transporter
DHPG	3,4-Dihydroxyphenylglycol
DNA	Deoxyribonucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid

E

ER	Endoplasmic reticulum
ETC	Electron transport chain

F

FDA	Food and Drug Administration

G

GABA	γ-amino butyric acid
GLU	Glutamate

H

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

I

IDO	Indoleamine-2,3-dioxygenase

IGR	Ionotropic glutamate receptor

J

JNK/SAPK	Jun N-terminal kinase/stress-activated protein kinase

K

KYNA	Kynurenic acid
KA	Kainic acid

L

l-MA	Methamphetamine
LSD	Lysergic acid diethylamide

M

MA	Methamphetamine
MAO	Monoamine oxidase
MDA	Malondialdehyde
MDMA	Methylenedioxymethamphetamine
MHPG	3-methoxy-4-hydroxyphenylglycol
MRI	Magnetic resonance imaging

N

NA	Noradrenalin
NE	Norepinephrine
NET	Noradrenalin transporter
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate

NO	Nitric oxide
NOS	Nitric oxide synthase

O

OFT	Open field test

P

PD	Parkinson's disease
PostND *x*	Postnatal day *x*
PPI	Prepulse inhibition
PreND *x*	Prenatal day *x*

Q

QA	Quinolinic acid

R

RNS	Reactive nitrogen species
ROS	Reactive oxygen species

S

Sal	Saline
s.c.	Subcutaneous
SERT	Serotonin (5-HT) transporter
SI	Social interaction
SIR	Social isolation reared
SOD	Superoxide dismutase
STD	Sexually transmitted disease

T	
TBA	Thiobarbituric acid
TDO	Tryptophan-2,3-dioxygenase
TH	Tyrosine hydroxylase
TH	Tryptophan hydroxylase
TNF-α	Tumor necrosis factor α
U	
USA	United States of America
V	
VMAT-2	Vesicular monoamine transporter-2
W	
WST-1	2-(4-iodophenyl)-2H-tetrazolium, monosodium salt
X	
XO	Xanthine oxidase

Addendum G - References

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