Methamphetamine exposure during pregnancy:

Neurodevelopmental consequences for the offspring

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Abstract

Methamphetamine (MA) abuse during pregnancy is a significant concern worldwide. Improving our understanding of the consequences of such exposure on the child is paramount and animal models provide an alternative method of investigating this growing issue. The present work aimed to investigate the developmental, behavioural and neurochemical effects of MA exposure on rat offspring following: 1) prenatal exposure to a range of doses; 2) prenatal and/or postnatal exposure; 3) prenatal intermittent and acute exposure and 4) prenatal exposure via different routes of administration, namely oral (gavage) and subcutaneous (sc). Prenatal and postnatal chronic exposure to MA leads to lower body weight gain and food intake in the mothers and these effects were transient and dose-dependent. Maternal care was compromised by MA when given prenatally by sc injection. Neonatal death was most significant after intermittent and acute prenatal exposure to MA and also after chronic exposure prenatally and postnatally. Chronic MA exposure prenatally and/or postnatally resulted in somatic developmental delays in pinna unfolding, fur appearance and eye opening, effects that were more pronounced following sc administration. Somatic development parameters such as ano-genital distance and body length were only altered by low dose MA or MA sc exposure. Behavioural impairments in surface righting, inclined plane and forelimb grip were only noted after chronic MA exposure prenatally and/or postnatally. All behavioural deficits observed were greater in the MA sc-exposed offspring. In adulthood, prenatal chronic exposure to MA lead to decreased anxiety-like behaviour and increased depressive-like behaviour in the elevated plus maze and forced swim test, respectively. Our neurochemical results showed that parturition and/or lactation and age increased oxidative stress compared to non-pregnant females however, prenatal

or postnatal MA had no significant effect on oxidative stress in the mothers or offspring. In conclusion, this research shows that MA exposure during pregnancy and lactation can lead to long-term developmental deficits in offspring. This developmental profile may offer guidance clinically as to what might be expected when a mother abuses this drug during pregnancy or lactation.

Author's Declaration

I hereby declare that the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland, Galway. The research is original and entirely my own with the following assistance:

- Adult behavioural testing in chapter 6 was assisted by Ms. Michelle Donlon, Ms. Sarah Horgan, Ms. Nina Wemken, Ms. Morgane Clarke, Ms. Kelly McHugh, Ms. Natalie Desanctis and Ms. Patricia Calcagno.

The thesis or any part thereof has not been submitted to any other institution in connection with any other academic award. Any views expressed herein are those of the author.

Signed: _____ Date: _____

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I have been lucky to have a large support network near, far and very far away; encouragement from across the world has driven me to become the person they knew I could be. My family has always been a constant reassurance that you can achieve whatever you put your mind to.

List of Publications and Conference Proceedings

Published Manuscripts

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McDonnell-Dowling, K. & Kelly, J. P. 2015. The consequences of prenatal and/or postnatal methamphetamine exposure on neonatal development and behaviour in rat offspring. *International Journal of Developmental Neuroscience,* 47, Part B**,** 147-156.

McDonnell-Dowling, K. & Kelly, J. P. 2015. Sources of variation in the design of preclinical studies assessing the effects of amphetamine-type stimulants in pregnancy and lactation. *Behavioural Brain Research,* 279, 87-99.

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Unpublished Manuscripts

McDonnell-Dowling, K. & Kelly, J.P. 2015. The consequences of prenatal or postnatal methamphetamine exposure on oxidative stress in the rat mother and offspring.

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Published Abstracts from Conference Proceedings

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McDonnell-Dowling, K. & Kelly, J.P. May 2015. Prenatal or postnatal exposure to methamphetamine impairs neonatal development and behaviour in rat offspring. *College of Medicine research day, NUI Galway*. Oral presentation.

McDonnell-Dowling, K., Donlon, M. & Kelly, J.P. May 2014. The consequences of methamphetamine exposure during pregnancy on maternal parameters, neonatal development and adult behaviour in rats. *College of Medicine research day, NUI Galway*. Oral presentation.

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McDonnell-Dowling, K. & Kelly, J.P. May 2015. Impact of prenatal or postnatal exposure to methamphetamine on adult behaviour in rats. *College of Medicine research day, NUI Galway*. Poster presentation.

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List of Abbreviations

2,3 DHBA; 2,3-Dihydroxybenzoic Acid

3-MT; 3-Methoxytyramine

4-HNE; 4-Hydroxynonenal

5-HIAA; 5-Hydroxyindoleacetic Acid

5-HT; Serotonin

6PGD; 6-Phosphogluconate Dehydrogenase

AA; Acetic Acid

ABTS; 2,2'-Azino-bis (3-Ethylbenzthiazoline- 6-Sulfonic Acid)

ACREC; Animal Care and Research Ethics Committee

ADMA; Asymmetric Dimethylated L-arginine

ALS; Amphetamine-Like Stimulants

AMP; Amphetamine

ANOVA; Analysis Of Variance

AP-1; Activator Protein 1

ATS; Amphetamine-Type Stimulants

AUC; Area Under Curve

BBB; Blood Brain Barrier

BSA; Bovine Serum Albumin

CAT; Catalase

CH; Cumene Hydroperoxide

CNS; Central Nervous System

COX; Cyclooxygenase

D; Day

DA; Dopamine

DAT; Dopamine Transporter

dH_2O; Distilled Water

DNA; Deoxyribonucleic Acid

DOPAC; 3,4-Dihydroxyphenylacetic Acid

DMI; Desipramine

DTNB; 5,5'-Dithiobis(2-Nitrobenzoic Acid)

DVD; Digital Video Disc

DVR; Digital Video Recorder

DZP; Diazepam

EPM; Elevated Plus Maze

F; Female

FC; Frontal Cortex

FDA; Food and Drug Administration

FRAP; Ferric Reducing Ability of Plasma

FST; Forced Swim Test

G6PD; Glucose-6-Phosphate Dehydrogenase

GABA; Gamma-Aminobutyric Acid

GCS; Glutamylcysteine Synthetase

GD; Gestation Day

GGT; Gamma-Glutamyltransferase

g-GTP; g-Glutamyltranspeptidase

Glrx; Glutaredoxin

GPx; Glutathione Peroxidase

GR; Glutathione Reductase

GSH; Glutathione

GSSG; Oxidised Glutathione

GST; Glutathione-Stransferase

H; Hour

H_2O_2; Hydrogen Peroxide

HCA; Home Cage Activity

HCL; Hydrochloride

HVA; Homovanillic Acid

IP; Intraperitoneal

IV; Intravenous

IZD; Inner Zone Duration

IZE; Inner Zone Entries

LD; Lethal Dose

M; Male

MA; Methamphetamine

MDA; Malondialdehyde

MDMA; Methylendioxymethamphetamine

Min; Minute

mRNA; Messenger Ribonucleic Acid

MWM; Morris Water Maze

N; No

NADPH; Nicotinamide Adenine Dinucleotide Phosphate

NE; North East

NF-κB; Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NIDA; National Institute on Drug Abuse

NIH; National Institutes of Health

NO; Nitric Oxide

No.; Number

NOS; Nitric Oxide Synthase

Nrf2; Nuclear Factor Erythroid 2–Related Factor

NS; Not Specified

NT; No Treatment

O_2; Oxygen

$O_2 \cdot^-$; Superoxide Radicals

OAE; Open Arm Entries

OAT; Open Arm Time

OECD; Organisation for Economic Co-operation and Development

OF; Open Field

$OH \cdot$; Hydroxyl Radicals

PBS; Phosphate-Buffered Saline

PC; Protein Carbonyls

PFC; Prefrontal Cortex

PND; Postnatal Day

PO; Oral

RHA; Roman High Avoidance

RLA; Roman Low Avoidance

ROS; Reactive Oxygen Species

SC; Subcutaneous

SD; Standard Deviation

S-D; Sprague-Dawley

SDS; Sodium Dodecyl Sulphate

Sec; Second

SFN; Sulforaphane

SNK; Student-Newman-Keuls

SOD; Super Oxide Dismutase

STR; Striatum

SW; South West

TBARS; Thiobarbituric Acid Reactive Substances

TH; Tyrosine Hydroxylase

TNF-α; Tumor Necrosis Factor-Alpha

UK; United Kingdom

US; United States

VEH; Vehicle

W; Wistar

Wk; Week

XO; Xanthine Oxidase

Y; Yes

Most abbreviations, other than commonly used expressions, are also defined at the first point of occurrence in the text.

Chapter 1:

Introduction I:

Sources of variation in the design of preclinical studies assessing the effects of amphetamine-type stimulants in pregnancy and lactation

McDonnell-Dowling, K. & Kelly, J. P. 2015. Sources of variation in the design of preclinical studies assessing the effects of amphetamine-type stimulants in pregnancy and lactation. *Behavioural Brain Research,* 279, 87-99.

Abstract

The prevalence of drug use during pregnancy has increased in recent years and the number of drug-exposed babies has therefore increased. In order to assess the risk associated with this there has been an increase in the amount of preclinical studies investigating the effects of prenatal and postnatal drug exposure on the offspring. There are many challenges associated with investigating the developmental and behavioural effects of drugs of abuse in animal models and ensuring that such models are appropriate and clinically relevant. The purpose of this review is to illustrate the variation in the design of preclinical studies investigating the effects of the amphetamine-type stimulants taken during pregnancy and/or lactation in animal models. Methamphetamine, methylendioxymethamphetamine and amphetamine were included in this review. The protocols used for exploring the effects of these drugs when taken during pregnancy and/or lactation were investigated and summarised into maternal experimental variables and offspring experimental variables. Maternal experimental variables include animals used, mating procedures and drug treatment and offspring experimental variables include litter standardisation, cross fostering, weaning and behaviours and parameters assessed. The findings in this paper suggest that there is a large diversity and little consistency among these studies and so the interpretation of these results may not be as clinically relevant as previously thought. For this reason, the importance of steering the preclinical studies in a direction that is most clinically relevant will be an important future recommendation. This will also allow us to be more confident in the results obtained and confident that the human situation is being replicated as closely as possible.

1.1 Introduction I

1.1.1 Drug abuse during pregnancy

Substance abuse in women typically occurs during the childbearing age (Anderson and Choonara, 2007) and reports have shown that over 90% of women that abuse drugs are aged between 15 and 39 years of age (Anderson and Choonara, 2007). It has been shown that out of all substances taken during pregnancy, tobacco and alcohol are the most commonly used (Arria *et al.*, 2006). However, the prevalence of other drug use during pregnancy is increasing and so the amount of drug-exposed babies is also increasing. Ostrea *et al.* (1992) performed drug screening of new-borns in a high-risk urban population by meconium analysis for commonly abused drugs (cocaine, opiates and cannabinoids) and showed that over 40% of babies tested positive for cocaine and morphine but that only 11% of the mothers had admitted to using such drugs during pregnancy. More recently, results from the U.S. national survey on drug use and health showed that 4% of pregnant women admitted to using drugs during pregnancy but again this figure is probably an underestimate of the actual prevalence of use (Substance Abuse and Mental Health Services Administration, 2013).

1.1.2 Amphetamine-Type Stimulants (ATS)

According to the World Health Organization, the ATS drugs are primarily made up of methamphetamine (MA) and amphetamine (AMP) but they also include methylendioxymethamphetamine (MDMA), methylphenidate, methcathinone, ephedrine, fenetylline and pseudoephedrine (World Health Organization, 2014). ATS use has become the most significant drug problem worldwide since the 1990s replacing that of cannabis, heroin and opium which had dominated the illicit drug

market up until a decade ago (United Nations Office on Drugs and Crime, 2013). When looking at the effects of prenatal ATS exposure in humans it is difficult, as it is for all drugs of abuse, as sample sizes are small, subjects can differ greatly with regards to dose and frequency of drug exposure and confounding variables such as other drug use are also common (Arria *et al.*, 2006). Therefore, in order to assess risk, there has been an increase in the amount of preclinical studies investigating the effects of prenatal and postnatal ATS exposure on the offspring (Figure 1.1). This review is confined to MA, AMP and MDMA use in a rodent model due to the predominant use of these three ATS drugs.

Methamphetamine

The U.S. National Survey on Drug Use and Health in 2012 showed that 12 million people (over 5% of population) aged 12 or more had used MA in their lifetime (Substance Abuse and Mental Health Services Administration, 2013). MA can easily cross the placental barrier during pregnancy (Anderson and Choonara, 2007) and therefore may put the offspring at risk. Early human studies found that children prenatally exposed to MA had increased stress, decreased arousal, movement disturbances and decreased school achievements (Chaikind and Corman, 1991, Smith *et al.*, 2006). Similar studies also showed that children had lower verbal and long-term spatial memory and lower visual motor integration (Chang *et al.*, 2004).

Many animal studies have aimed to elucidate the short and long-term effects of prenatal MA exposure. Behavioural consequences seen in rats include decreased pre-pulse inhibition and increased startle reflexes (Slamberova *et al.*, 2006). Adverse effects that have been reported include cleft palate, retinal eye defects, delayed motor

development and physical growth (Cho *et al.*, 1991, Acuff-Smith *et al.*, 1992, Yamamoto *et al.*, 1992, Cabrera *et al.*, 1993, Weissman and Caldecott-Hazard, 1995). One study using a rat model interestingly found that the adverse effects found after prenatal MA exposure were even passed to the next generation of offspring such as poorer performance in righting reflex and bar-holding tests (Slamberova *et al.*, 2007).

Amphetamine

In a similar fashion to MA, AMP can cross the blood-brain barrier easily and stimulates the CNS by acting as a sympathomimetic drug, as does MA (Kraemer and Maurer, 2002). Early human studies by Larsson (1980) showed that women who had taken AMP throughout their pregnancy had complications such as preeclampsia and higher incidences of preterm births with a quarter of these mothers delivering preterm. Congenital malformations include uro-genital anomalies, pilonidal sinus, limb deformity and ear abnormalities (Golub *et al.*, 2005, Nelson and Forfar, 1971). These malformations were seen after various AMP exposures including the whole pregnancy, the first trimester and even after the first 14 days of pregnancy.

Malformations that have been observed in rat embryos after AMP exposure include neural tube defects, microcephaly and incomplete rotation of the body axis and tortuous spinal cord (Yamamoto *et al.*, 1998). Behavioural alterations have also been reported after *in utero* AMP exposure. Tan (2003) showed that AMP exposure from gestation day (GD) 8 until birth, increased startle amplitude in the offspring and showed less inhibition for the prepulse startle trials after an AMP challenge. This

indicates a different profile of behavioural reactivity in adulthood after this psychopharmacological challenge.

Methylendioxymethamphetamine

MDMA acts as both a stimulant and a hallucinogenic drug and it is usually ingested in pill or tablet form (United Nations Office on Drugs and Crime, 2013). It is commonly sold as 'ecstasy' in pill form which contains other psychoactive substances (United Nations Office on Drugs and Crime, 2013). Infants who are exposed to MDMA during pregnancy show poor motor quality and lower milestone attainment at four months of age (Singer *et al.*, 2012). These retardations have also been reported after MDMA exposure during just the first trimester of pregnancy and a recent drugs and infancy study showed that the degree of psychomotor deficit was directly correlated to the dose of MDMA used (Parrott *et al.*, 2014).

Barenys *et al.* (2010) showed that male rat offspring had increased DNA damage in sperm and interstitial oedema in testes, decreased sperm motility and delayed preputial separation onset after MDMA exposure *in utero* and during lactation. Developmental delays in the offspring have also been shown, where incisor eruption, eye opening and ability to perform negative geotaxis tests have all been delayed after exposure in the last week of pregnancy (Heuland *et al.*, 2010).

1.1.3 Animal models

The use of animal models has enabled us to understand the neurodevelopmental and behavioural consequences of drug exposure during pregnancy and breastfeeding. There are many challenges associated with testing the developmental effects of drugs

of abuse in animal models and ensuring these models are appropriate. Before designing an experiment with animal models, the experimenter must take important factors into consideration including the species, strain, and age of the animals, the sample sizes used, housing conditions, the drug treatment regime (including the dose, route of administration, duration and frequency), the controls used, the selected behavioural tests and the parameters to be assessed. For both humans and animal models, the neurodevelopmental timeline has been well documented i.e. from conception through the neonatal period (Clancy *et al.*, 2001, Clancy *et al.*, 2007). For the purpose of developmental studies it is important to try and represent the human scenario as closely as possible and to ensure that the timing of the exposure is realistically representative to humans (Thompson *et al.*, 2009a). Of course the closest model of human brain development is non-human primates but these are an expensive option and are not ideal for rapid mechanistic discoveries (Thompson *et al.*, 2009a). The timeline and correlation between rat and human pregnancies has been carefully studied. This allows us to correlate the stages of foetal development in the rat to that of humans. In contrast to humans, neurodevelopment in rats occurs during pre- and postnatal periods, and so one can easily examine effects of drug administration over different exposure periods that are relevant to human pregnancy (Daston *et al.*, 2004). In order to mimic the clinical scenario, as many parameters as possible should be kept the same (e.g. route of drug administration, timing of drug exposure) and other parameters should be translated for the animal model (e.g. doses of drug used). Guidelines for performing a toxicity and reproductive study have been published by the FDA and these exist to ensure consistency among studies (Collins *et al.*, 1999b).

1.1.4 Review methods

The search terms 'Methamphetamine, Pregnancy, Animals', 'Amphetamine, Pregnancy, Animals' or 'MDMA, Pregnancy, Animals' were entered into PubMed search engine. Between 1960 and 2013, 883 articles were published in this area (Figure 1.1). Among these articles 112 were relevant in that they involved ATS drug exposure during pregnancy or lactation (MA, 73; AMP, 17; MDMA, 22).

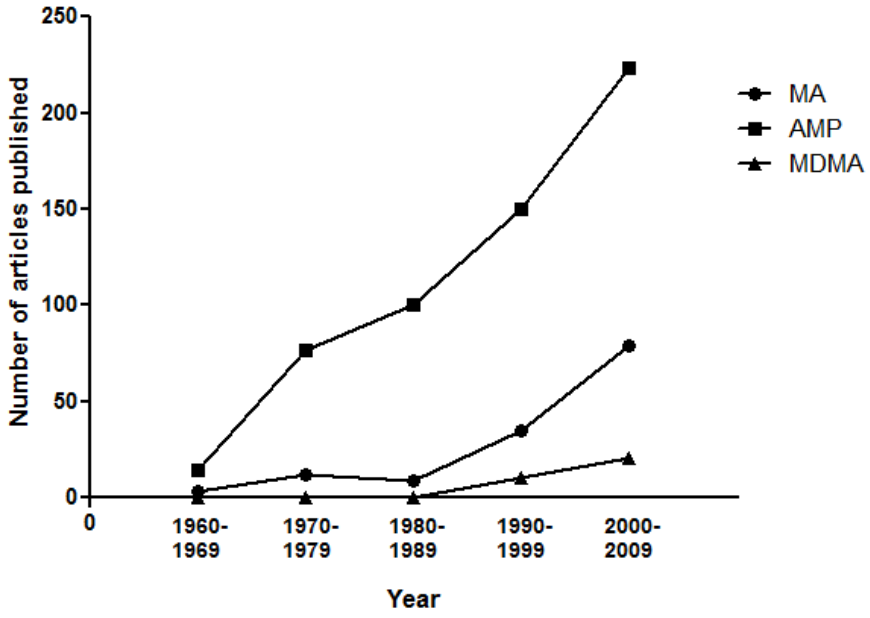

Figure 1.1: ATS Papers published. Total number of articles returned when 'Methamphetamine, Pregnancy, Animals', 'Amphetamine, Pregnancy, Animals' or 'MDMA (or methylendioxymethamphetamine), Pregnancy, Animals' was entered into PubMed search engine.

1.1.5 Aims

The purpose of this review is to illustrate the variation in methods employed when studying the effects of MA, AMP and MDMA when taken during pregnancy and/or lactation in animal models.

1.2 How does prenatal/neonatal ATS exposure vary?

Consistency with experimental variables is imperative to ensure the results seen are due to the neurotoxicity of the drug and nothing else. The protocols used for exploring the effects of ATS when taking during pregnancy or lactation can vary in several areas. By looking separately at the maternal experimental variables and the offspring experimental variables, we can categorise these variables accordingly (Figure 1.2).

1) Maternal experimental variables
    a. Animals used
    b. Mating procedure
    c. Drug treatment
2) Offspring experimental variables
    a. Litter standardisation and cross fostering
    b. Behavioural tests and developmental parameters assessed

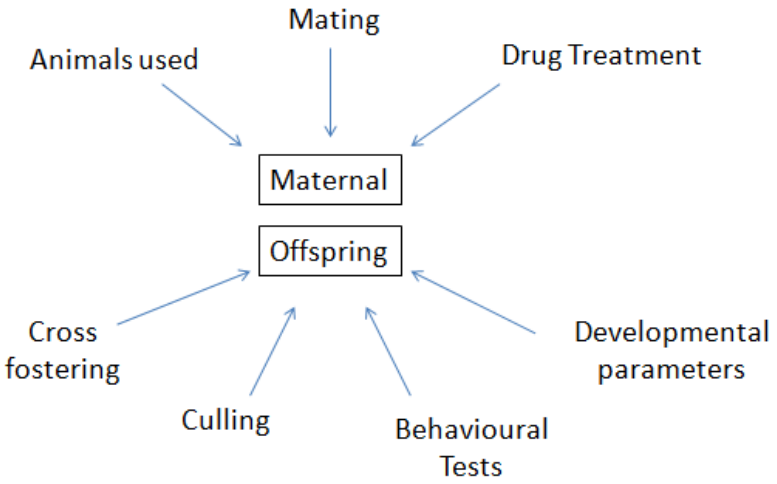

Figure 1.2: Experimental variables. Chart depicting the experimental variables that exist in ATS drug studies.

1.2.1 Maternal experimental variables

Developmental toxicity studies enable us to look at the impact of a test substance on offspring development after exposure during the gestation period (Collins *et al.*, 1999a). These studies can provide vital information on how a test substance can negatively affect development, or worse, if the toxicity of the drug can lead to neonatal death (Collins *et al.*, 1999b). The design of these studies needs to be carefully planned and many maternal experimental factors need to be considered, including animal choice, mating procedures and drug treatment factors.

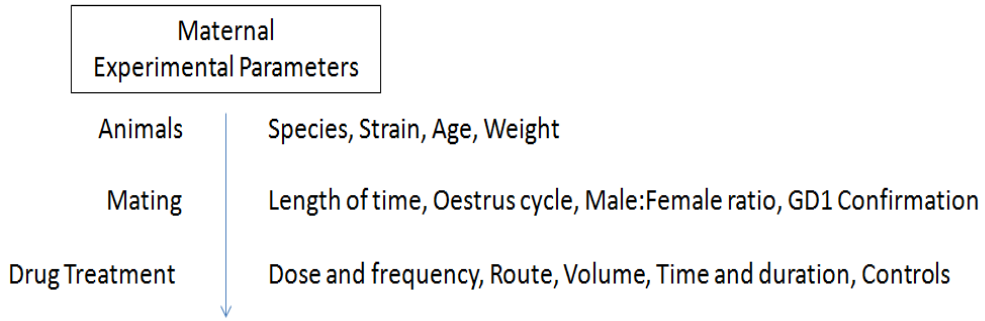

Figure 1.3: Maternal experimental variables. Chart depicting the maternal experimental variables that exist.

Animals

When selecting a test subject the rat has shown to be the most commonly used species for studies investigating reproduction and fertility effects (U.S. Environmental Protection Agency, 1998). In particular, for developmental toxicity studies, the rat is the ideal species due to its high fertility rate, ease of breeding and a short gestation length of three weeks (Collins *et al.*, 1999b). In comparison to mice, rats are less susceptible to stress effects (Collins *et al.*, 1999b) and they provide reliable results that can be translated to humans. In ATS developmental studies, there

is a higher incidence of rat use, with over 80% of these studies using rat as the test subject as opposed to the mouse (6-19%) and this is similar across all three ATS drugs (i.e. MA, MDMA, AMP, Table 1.1).

Drug	Rats	S-D	W	NS	Mice	No. of papers
MA	88%	36%	64%	-	12%	73
MDMA	81%	76%	24%	-	19%	22
AMP	94%	56%	25%	19%	6%	18

Table 1.1: Species and strains of animals previously used. Different species and strains of animals used for ATS studies. Data expressed as percentage of papers employing each parameter. S-D, Sprague-Dawley; W, Wistar; NS, Not Specified.

Another factor to consider is the strain of animal used and this has been different between ATS drugs. The main rat strains previously used are Wistar and Sprague-Dawley rats however, some early animal studies have failed to identify which strain was used with 19% of the AMP studies failing to disclose this information (Ramirez *et al.*, 1979, Adams *et al.*, 1982). In general, Wistar rats are more active and Sprague-Dawleys are known for their ease of handling and calmness and in general both strains are commonly used in all preclinical studies. However, between these strains there should not be a great difference between them for the purpose of studying ATS drug use during pregnancy. Although Sprague-Dawley are noted to have excellent reproductive performance and the Wistar rats generally tend to have smaller litters which may make inter-litter comparisons difficult (Harlan Laboratories, 2014) both strains have been shown to be successful models for this type of preclinical study.

For reproductive studies it is recommended that the naïve parental animals are between 5 and 9 weeks old when exposure to the test substance begins (Collins *et al.*, 1999b) however, breeding for rats usually begins at 3 months of age (Wolfensohn and Lloyd, 2007) and it is vital, naturally, that the rats are sexually mature at time of mating. For this reason, at the time of mating male rats should weigh approximately 300-350 g and females should weigh approximately 225-250 g (Wolfensohn and Lloyd, 2007). In relation to ATS studies, some studies looking at the effects of MA have used females that are 150-175 g (Grace *et al.*, 2010, Williams *et al.*, 2003, Vorhees *et al.*, 2005) which would be roughly two months of age (Table 1.2). This may be too young to breed female rats as puberty usually only occurs around this time (Wolfensohn and Lloyd, 2007), therefore the pregnancy success rate may be reduced. There is a large variance with the weight and age of females used between ATS studies. In a study by Williams *et al.* (2004) investigating the effects of prenatal MA, females weighed 150-175 g at the time of mating whereas Slamberova *et al.* (2011b) and Bernaskova *et al.* (2011) used females between 250-300 g. This weight difference of 150 g corresponds to an age difference of 40+ days and highlights the importance and need for standardisation across studies that are examining reproductive toxicity. Although there is no evidence to suggest that there is an effect of age we cannot be sure of this and so this may warrant further investigation into the various ages that are used in these ATS studies. Again many studies have failed to disclose information regarding the weight or age of the animal (Barenys *et al.*, 2010, Flores *et al.*, 2011, Cui *et al.*, 2006) and so it is unclear if the animals are of uniform size and weight (Table 1.2). This section highlights the importance of stating not only the weight of the animals used but also the age of these animals.

Weight/Age	MA	MDMA	AMP
Unknown weight	43%	95%	94%
150-175 g	4%	0%	0%
180-270 g	1%	0%	0%
200-250 g	3%	0%	0%
225-275 g	0%	0%	6%
250-300 g	49%	5%	0%
Unknown age	84%	84%	90%
5 – 7 weeks	2%	5%	0%
8 - 10 weeks	13%	6%	0%
12 – 14 weeks	0%	0%	10%
15 - 16 weeks	1%	5%	0%

Table 1.2: Weights and ages of animals previously used. Different weights and ages of animals used for ATS studies. Data shown as percentage of papers.

Mating

The administration of ATS drugs during pregnancy or lactation and the timing of dosing is specific to each study. In order to begin dosing on a specific GD, knowing the exact time of copulation (i.e. insemination) is vital. In order to confirm this vaginal smears can be performed or the presence of vaginal plugs can be examined (U.S. Environmental Protection Agency, 1998). The use of vaginal plugs as a confirmation of mating can be problematic as there is a potential for vaginal plugs to be formed without successful mating (Hood, 2005). The use of vaginal smears can also be associated with stress due to the handling and restraint of the animal but if the rat is treated gently, over time the female rats will habituate to the handling (Marcondes *et al.*, 2002). All females should be treated in the same way and all females should be smeared the same amount of times. The vaginal smear is the

favoured protocol for verifying that mating has occurred as if sperm is detected in the smear then one can confirm that insemination has occurred.

Regardless of which protocol is employed, each female should be examined daily for these parameters during mating and day 0 of pregnancy is defined as the day in which a vaginal plug is observed or sperm is present in the vaginal smear (Collins *et al.*, 1999a, U.S. Environmental Protection Agency, 1998). However, although a lot of studies would use this as an indication of pregnancy day 0 (Hruba *et al.*, 2009a, Eun *et al.*, 2010, Skelton *et al.*, 2009, Adori *et al.*, 2010) some studies have counted this as pregnancy day 1 (Pometlova *et al.*, 2009, Slamberova *et al.*, 2007, Tavares *et al.*, 1996, Nasif *et al.*, 1999, Tan, 2003, Won *et al.*, 2002). This poses a problem in that the beginning of dosing may be a day early or a day late and therefore the animals may be receiving an extra dose of the drug or may be missing a dose of the drug. For example, in a study by Won *et al.* (2002) the presence of a vaginal plug indicated the first day of pregnancy and the animals were dosed with MDMA from GD 6-13. However, if the presence of the plug is in fact GD 0 then these animals were actually being dosed during GD 5-14. Or in other cases when one dose is being administered on one specific GD, for example, 40 mg/kg MA via subcutaneous injection (sc) on GD 14 when the day of vaginal plug is GD 1 (Won *et al.*, 2001) or 40 mg/kg MA via intraperitoneal injection (ip) on GD 17 when the day of vaginal plug is GD 1 (McCallum *et al.*, 2011). Although these studies are trying to pinpoint a particular point of development, because they have not considered the presence of a vaginal plug as GD 0 then dosing has actually taken place on GD 13 and GD 16, respectively.

Drug treatment

Dose and frequency

The first studies that looked at MA and AMP exposures during pregnancy began in the early 1970s (Martin, 1975) and the doses employed here were taken from earlier adult behavioural studies with amphetamine (Teitelbaum and Derks, 1958). Tables 1.3.1, 1.3.2, 1.3.3 show the range of doses used in all 73 MA studies, 22 MDMA studies and 18 AMP studies: these are representative of the range of ATS doses previously and presently used in the literature.

Strain	Weight/Age	Total daily dose (mg/kg)	Route	No. of days of drug admin	Dosing period	No. of papers
S-D	250–300 g	5	sc	63	3 weeks + GD1 - PD 21/22	1/73
W	250–300 g	5	sc	63	3 weeks + GD1 - PD 21/22	9/73
W	10 weeks	1	ip	22	GD 1 - 22	1/73
S-D	NS	2	sc	21	GD 1 - 21	1/73
W	5 weeks	1,2,3 or 4.5	sc	14	GD 7 - 20	1/73
S-D	225-250 g	2, 6, 10	sc	21	GD 1 - 21	1/73
S-D	250–275 g	4	sc	21	GD 1 - 21	1/73
W	8 weeks	5	sc	15	GD 8 - 22	6/73
W	250–300 g	5	sc	22	GD 1 - 22	17/73
S-D	200–250 g	5	sc	22	GD 1 - 22	1/73
S-D	10 weeks	5	sc	11	GD 10 - 20	1/73
S-D	180 to 270 g	5.6, 10, 13.2, or 17.8	sc minipumps	16	GD 7 - 22	1/73
S-D	NS	10	ip	6	GD 8, 10, 12, 14, 16, 18	1/73
S-D	250–275 g	10	sc	42	GD 1 - PND 21	2/73
W	NS	80	po	42	GD 1 - PD 21	1/73
S-D	NS	10	sc	8	GD 13 - 20	1/73
S-D	NS	10, 20, 30, 40	sc	6	GD 7 - 12 or GD 13 - 18	1/73

Strain	Weight/Age	Total daily dose (mg/kg)	Route	No. of days of drug admin	Dosing period	No. of papers
S-D	151-175 g	2.5, 5, 10, 20	sc	10	PND 11 - 20	1/73
S-D	NS	4	NS	1	PND 14/21/ 28/ 56	1/73
W	NS	5 and 10	sc	7	PND 4 - 10	1/73
W	8 weeks	10	sc	29	PND 1 - 29	1/73
S-D	151-175 g	10, 20, 40	sc	10	PND 21-30/ 31-40/ 41-50/ 51-60	1/73
W	250–300 g	20	sc	21	PND 1-7/ 1-14/1-30	1/73
S-D	151-175 g	20	sc	10	PND 11 - 20	1/73
S-D	151-175 g	40	sc	10	PND 11 - 20	1/73
S-D	NS	40	sc	5	PND 11 - 15	1/73
S-D	NS	40	sc	10	PND 11 - 20	3/73
S-D	NS	40	sc	1	PND 11	1/73
S-D	NS	40, 100	sc	10	PND 1-10/6-15	1/73
S-D	NS	40, 60, 80, 100	sc	5	PND 11 - 15	1/73

Table 1.3.1: MA prenatal studies. The range of doses employed in MA prenatal studies. n=73 MA papers. S-D, Sprague-Dawley; W, Wistar; NS, Not Specified; GD, Gestation Day; PND, Postnatal Day.

Strain	Weight/Age	Total daily dose (mg/kg)	Route	No. of days of drug admin	Dosing period	No. of papers
S-D	9 weeks	0.5, 5, 10	sc	30	3 weeks + GD 1 - PND21	1/22
S-D	NS	2.5, 10	po	13	GD 6-18	1/22
W	NS	10	sc	8	GD 13 - 20	2/22
W	NS	15	ip	3	GD 4, 11 and 18	1/22
S-D	NS	30	sc	7	GD 14 - 20	3/22
W	250 g	40	sc	4	GD 14 - 17	1/22
S-D	NS	40	sc	4	GD 15-18 and PND 10-14/ 15-18/20-23/25-28/30-33	1/22
S-D	NS	10, 20, 40	po	1	PND 10/40/70	1/22
S-D	NS	40	sc	5	PND 11 - 15	1/22
S-D	NS	40	sc	10	PND 11 - 20	6/22

Table 1.3.2: MDMA prenatal studies. The range of doses employed in MDMA prenatal studies. n=22 MDMA papers. S-D, Sprague-Dawley; W, Wistar; NS, Not Specified; GD, Gestation Day; PND, Postnatal Day.

Strain	Weight/Age	Total daily dose (mg/kg)	Route	No. of days of drug admin	Dosing period	No. of papers
S-D	13 weeks	2, 5	po	51	30 days + GD 1 - 21	1/18
NS	NS	0.5	sc	21	GD 1 - 21	1/18
S-D	NS	0.5	sc	21	GD 1 - 21	1/18
NS	NS	0.5, 1, 2	sc	4	GD 12 - 15	1/18
S-D	NS	0.5, 2	sc	4	GD 12 - 15	2/18
S-D	NS	1	sc	11	GD 11 - 21	1/18
S-D	NS	2	sc	15	GD 7 - 21	1/18
S-D	NS	2, 5	sc	4	GD 11 - 14	1/18
S-D	NS	3	sc	4	GD 12 - 15	1/18
RHA and RLA	NS	3	sc	14	GD 7 - 20	1/18
W	NS	4	sc	15	GD 8 - 22	1/18
W	14 weeks 225-272 g	5, 10	sc	15	GD 8 - 22	1/18
W	NS	10	sc	15	GD 8 - 22	2/18
S-D	NS	5, 15, 25	po	6	PND 4 - 9	2/18

Table 1.3.3: AMP prenatal studies. The range of doses employed in AMP prenatal studies. n=18 AMP papers. S-D, Sprague-Dawley; W, Wistar; RHA, Roman High Avoidance; RLA, Roman Low Avoidance; NS, Not Specified; GD, Gestation Day; PND, Postnatal Day.

Each of these doses can be classified depending on the amount of drug given and the frequency in which it is given and this has been shown in table 1.4. Doses of ATS drugs can be classed as neurotoxic, toxic and pharmacological. The purpose of these preclinical studies should be to investigate the margins of safety of these uncontrolled drugs at doses that are typically abused in humans rather than at doses that we know to be neurotoxic.

Dose Classification	Dose Range	Outcomes	Comments
Neurotoxic	5+ mg/kg	CNS deficits Maternal death Offspring death	Resemble extremely high toxic doses
Toxic	2-4 mg/kg	Marked maternal BW decrease Marked developmental delay in offspring	Resemble high pharmacological doses
Pharmacological	0.25-2 mg/kg	Minimal maternal effects Minimal offspring effects	Resemble doses used in pharmacological studies

Table 1.4: Classification of previously used preclinical doses for ATS drugs.

A variety of doses have been explored in prenatal and neonatal ATS exposure studies however, many of these studies fall into the neurotoxicological doses. When we look to the clinical situation we see many patterns of exposure (Table 1.5) across each trimester of pregnancy but it has been shown that the most common frequency of MA use, for example, in pregnancy is generally almost every day/daily (Della Grotta *et al.*, 2010) and although there are a wide range of doses that are abused by

these females (Table 1.5), Golub *et al.* (2005) reports that the most common MA dose is around 30 mg.

	First Trimester	Second Trimester	Third Trimester
Dose	0.02–10.5 g	0.02–9.0 g	0.02–7.0 g
Frequency	0.1–7 days	0.1–7 days	0.1–7 days

Table 1.5: Range of doses and frequencies of use for MA in pregnant females. Data are expressed in range of grams/use and range of days/week. (Adapted from Della Grotta *et al.*, 2010)

The minimum doses of AMP, MDMA and MA examined in preclinical studies have been 0.5, 0.5 and 0.625 mg/kg (Williams *et al.*, 2004, Ramirez *et al.*, 1979, Barenys *et al.*, 2010) and the maximum doses have been 100, 20 and 40 mg/kg, respectively. (Wong *et al.*, 2008, Buttar *et al.*, 1996, Kaizaki *et al.*, 2014). However, by increasing the frequency of doses we can see that some studies have also reached a daily dose of 100 mg/kg by administering 25 mg/kg four times daily (Vorhees *et al.*, 2009). This extremely high dose would be considered a neurotoxic dose and it does not give us any real information on what harm can occur at a clinically used dose. The most common MA dose used is 5 mg/kg with 48% of the MA preclinical studies looking at this dose (Slamberova *et al.*, 2011b, Siegel *et al.*, 2010, Schutova *et al.*, 2009a, Pons *et al.*, 2007, Williams *et al.*, 2004). While it is not easy to directly match doses in humans and animals, methods have been developed for estimating these (Skelton *et al.*, 2008). For example, the use of an allometric scale takes into account the weight and body surface area (Km factor) of the human and animal (Reagan-Shaw *et al.*, 2008). By using this method by Reagan-Shaw *et al.* (2008) to translate doses from rat to human, the common dose of 5 mg/kg in a rat becomes 50 mg in a

pregnant human female (weighing approx. 70 kg, Table 1.6). Although this dose gives us valuable information relating to a female that is abusing a higher dose of MA there is still a paucity of studies looking at lower doses of ATS drugs.

Human Dose (mg)	Rat Dose (mg/kg)
30	2.5
120	10
480	40
960	80
1200	100

Table 1.6: Translation of human ATS doses. Conversion of human doses to rat doses using an allometric scale (Reagan-Shaw *et al.*, 2008). Human dose (mg) based on a 70 kg human.

The frequency of dosing on each day varies between each study. The most common frequency is one dose per day (Flores *et al.*, 2011, Barenys *et al.*, 2010, Inoue *et al.*, 2004) but some studies have used two, three and even four doses per day. This has been used to repeat the same dose throughout the day to achieve a higher daily dose (Vorhees *et al.*, 2009, Colado *et al.*, 1997, Tavares and Silva, 1996), as a neurotoxic regime (Vorhees *et al.*, 2005) or to divide out the full dose over the day (Pons *et al.*, 2007, Thompson *et al.*, 2009b, Tavares and Silva, 1996). For example, as AMP has a half-life of 14 h in humans, by administering two doses per day the experimenter aims to maximise the time that the drug and its metabolites remain in the animal and therefore mimic the clinical scenario (Martin, 1975). The advantage of using one dose a day is that regardless of the route of administration the animal will not be as stressed receiving one dose rather than being dosed three or four times a day.

However, by administering the drug throughout the day one can look at a higher daily dose without running the risk of giving the animal an overdose or reaching the LD_{50}.

Route

The route of administration is a very important factor to consider when designing a study to look at prenatal and/or postnatal drug exposure. A route that most closely mimics the human route of exposure should be selected to administer the drug in question (Collins *et al.*, 1999a) and one that ensures the animal is receiving the entire dose. Among the ATS studies, the most common route of administration is subcutaneous injection (sc) with 97 of the ATS studies (86%) using this route (Grace *et al.*, 2010, Vorhees *et al.*, 2009, Tan, 2003, Heuland *et al.*, 2010) (Figure 1.4). This route ensures that the full dose is administered, for example, compared to a drug being administered in drinking water which can be variable. However, with this type of route the relationship or connection to the clinical circumstance is lost as there is no data to show that pregnant women administer drugs to themselves via the sc route. The ip route has been used in few MA and MDMA studies (Wong *et al.*, 2008, Siegel *et al.*, 2010, Inoue *et al.*, 2004, Adori *et al.*, 2010) (Figure 1.4). The drug is absorbed more rapidly this way compared to sc but again we do not have the clinical relevancy with this route. Osmotic minipumps (sc) have been employed in MA studies which involve a chronic infusion of the drug and maintain a steady-state exposure (White *et al.*, 2009). Although this route achieves a drug concentration that would be similar to humans the clinical relevancy is being lost and it does not mimic what is truly occurring in the clinical scenario. According to the FDA guidelines for reproductive and developmental studies, the drug in question should be delivered by

a route that most closely approximates the human route of exposure and therefore an oral or inhaled route of administration should be employed (Collins *et al.*, 1999a). In humans, and likewise in pregnant females, ATS drugs are most commonly taken as a bolus dose by either ingesting the drug in tablet or powder form or smoking the drug (U.S. Department of Health and Human Services, 2006). To our knowledge, there are no studies that have investigated the effects of ATS use during pregnancy using inhalation as the route of administration. This may be as this method can be quite expensive and the setup is quite elaborate in that concealed individual cages would be necessary. The use of an oral route (po) of administration is rare (Figure 1.4) and has been employed by administering the drug in the drinking water (Tonge, 1973, Monder, 1981) or oral gavage (Buttar *et al.*, 1996, Broening *et al.*, 1994). By dissolving the drug in drinking water the drug is delivered effectively however, some problems are associated with this method. For example, it is very difficult to know at what time the drug is been taken by the animal and if the drug is being ingested constantly over the day or in short durations of 1-2 times daily. In this case, each animal may be receiving different dosing regimens. The delivered dose for each animal can also vary incredibly depending on how much they consume and one cannot be confident that a particular drug group have all received the same treatment and concentration of drug. Oral gavage, as a route of administration, can overcome these limitations in that the drug is still delivered effectively but one can control the time of exposure, the frequency and volume of drug exposure. This method is by far the most suitable and the most clinically relevant. Although it has been employed in many studies looking at MDMA exposure (Cho *et al.*, 2008, Kaizaki *et al.*, 2014, Eun *et al.*, 2010) and few studies looking at AMP exposure (Smith and Chen, 2009, Smith and Chen, 2010, Buttar *et al.*, 1996), oral gavage has never been employed for

studies looking at MA exposure despite its clinical relevancy. Further investigation into the different routes of administrations used would be very beneficial and a comparison study would tell us if there are any differences between routes and if the amount of drug that reaches the foetus is in fact similar between all routes.

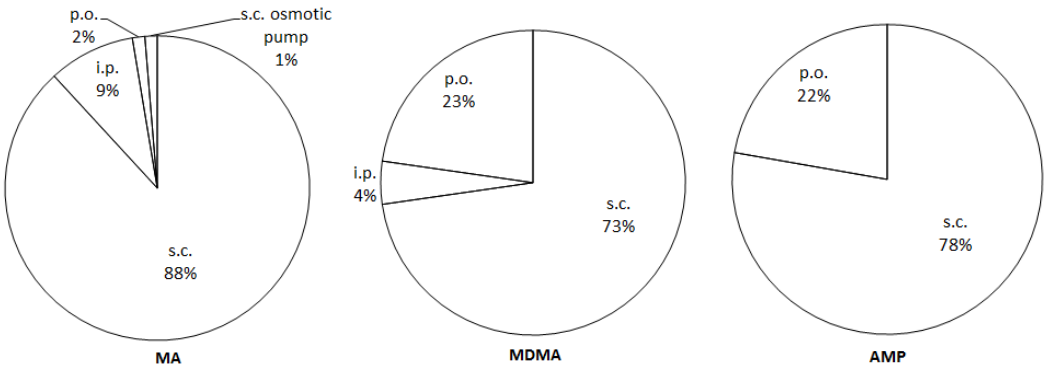

Figure 1.4: Routes of administration. Pie chart depicting the different routes of administration used across ATS studies.

Time and duration of exposure

Women who use ATS drugs can range from infrequent recreational drug users to heavy dependent drug users (National Drug & Alcohol Research Centre, 2007). For recreational users, mothers have admitted to taking an ATS drug once a month or 2-3 times per month during pregnancy whereas chronic users admitted to taking the drug 1-2 times per week, 3-4 times per week or almost every day during the entire pregnancy (Della Grotta *et al.*, 2010). These patterns of use however, can change depending on the ATS drug. For MDMA, the pattern of use during pregnancy has shown to change over the course of the pregnancy. Singer *et al.* (2012) showed that among pregnant females their use of MDMA dropped steadily as they progressed through each trimester (3^{rd} trimester dose <0.8% of 1^{st} trimester dose). However, although some females have also shown to decrease their MA use as they progress

into their pregnancy, a large-scale study in the U.S. showed that out of 922 MA-using pregnant females, 25.7% stayed on a low/moderate dose, 29.3% stayed on a consistently high dose and 9.4% of mothers actually increased their MA dose (Della Grotta *et al.*, 2010). As there is no standard definition for ATS use patterns during pregnancy there are many different dosing regimens during gestation that have previously been employed in preclinical ATS studies (Table 1.7). These regimens either mimic a certain exposure pattern that is seen in pregnant females or targets a certain point of development in the offspring to see the effect of drug exposure on this developmental parameter. Among these different regimens, dosing throughout the entire gestation period is the most commonly used duration of exposure (Schutova *et al.*, 2009a, Yamamotova *et al.*, 2011, Bubenikova-Valesova *et al.*, 2009, Ramirez and Carrer, 1983) (Table 1.7). This means that exposure to the drug begins on the first day of gestation (GD 0/1) and continues throughout until the last day of gestation or birth (usually GD 21/22). However, the length of the gestation period can be different between each dam, and so some studies that used this pattern of exposure have administered an additional one to two drug injections (Slamberova *et al.*, 2005b, Slamberova *et al.*, 2005c) to ensure that the pregnant dams did not experience withdrawal prior to delivery. A dosing regimen like this relates to the clinical situation where the mother is abusing MA everyday throughout pregnancy. This is consistent with a study by Della Grotta *et al.* (2010) that showed 55% of pregnant MA users did not change their pattern of use of MA over the entire course of their pregnancy. The other 45% of these users either decreased their use over time (35%) or increased their use over time (10%).

Dosing Period	% of papers
3 weeks + GD 1 - PND 21/22	10
GD 1 - 22	21
GD 1 - PND 21	3
GD 7 - 20	12
GD 12 - 15	6
GD 13 - 20	6
PND 4 - 10	3
PND 11 - 20	11

Table 1.7: Dosing regimens. The most common dosing regimens that have previously been employed in ATS studies. GD, Gestation Day; PND, Postnatal Day.

To the best of our knowledge, there is no data for pregnant females abusing ATS drugs on their use of these drugs before and after pregnancy. It is unknown if these females are previous ATS users that become pregnant and continue using the drug or if they begin use during pregnancy. Some studies (10% of ATS studies, Table 1.7) have therefore included a dosing period that occurs before conception for example an animal that receives three weeks of dosing before gestation, three weeks of dosing during gestation and three weeks of dosing after gestation (Slamberova *et al.*, 2010a, Hruba *et al.*, 2010, Pometlova *et al.*, 2009).

With regards to the aforementioned study (Della Grotta *et al.*, 2010), it is unknown if the 35% of women that decreased their MA use during pregnancy were able to maintain this and become abstinent or relapse and return to their original patterns of use during the lactation period. For this reason, the postnatal period is a potential time of drug exposure for the offspring as ATS drugs can pass easily to the offspring

via the breast milk. Again, there is no standard definition for ATS use patterns during the breastfeeding period and so many different dosing regimens during lactation have previously been investigated in preclinical ATS studies. Exposure to an ATS throughout the entire neonatal period has only been investigated using MA (Gomes-da-Silva *et al.*, 2000, Gomes-da-Silva *et al.*, 2004) and this exposure regimen has never been studied for MDMA or AMP. This means that exposure to the drug begins on the first postnatal day or day of birth (PND 1) and continues throughout until the end of the adolescent period (PND 29/30). However, in these investigations and other dosing regimens in the postnatal period, we are seeing deviations from the clinical situations whereby the drug is being administered directly to the offspring during the postnatal period (Schaefer *et al.*, 2008, Williams *et al.*, 2004, Koprich *et al.*, 2003, Smith and Chen, 2009). Some studies that used this method date back to the early ATS studies (Broening *et al.*, 1994) however, many of the more recent papers (within the last 10 years) have employed this method and in this area, 99% of the ATS studies that have looked at postnatal exposure have administered the drug directly to the offspring during the postnatal period. Some of these studies have aimed to target a specific postnatal developmental period in rats, for example a period that is analogous to the human third trimester (PND 11-20), and so administration directly to the pup may be suitable. However, the dose that a pup would receive via the breast milk is far from the doses that are being administered in these studies. To the best of our knowledge there is only one ATS study that has examined the effects of ATS postnatal exposure by administering the drug to the mother (Rambousek *et al.*, 2014). As ATS-exposed offspring are actually exposed to the drug via breast milk during the lactation period in humans then the same should also be done in preclinical studies rather than injecting the offspring directly.

Appropriate control groups

As with all other preclinical research, studies investigating the effects of prenatal and/or postnatal ATS exposure use a comparable control. The most common control used in these studies usually receives the vehicle that the active compound is delivered in using the same route of administration, at the same volume and at the same time of day (Inoue *et al.*, 2004, Barenys *et al.*, 2010, Nasif *et al.*, 1999). Some studies also use an additional non-treatment group in order to compare the effects of the stress associated with the route of administration alone e.g. injection and/or restraint (Slamberova *et al.*, 2011a, Yamamotova *et al.*, 2011, Smith and Chen, 2009). However, this non-treated control is not an appropriate control on its own and should only be used as an additional control group. Schutova *et al.* (2009b) previously used this control alone but by not having a sc injected control group one cannot be sure the effects observed are due to the drug alone and not to the stress associated with sc administration. Another parameter that has been taken into account with regards controls is the potential anorectic properties of ATS drugs, and in particular MA. A litter that is born to a mother that had nutritional deficits during her pregnancy may be underweight when born and may also have developmental delays as a result. In order to overcome this, the use of pair-fed controls have been employed (Pons *et al.*, 2007, Melo *et al.*, 2006, Acuff-Smith *et al.*, 1996, Tan, 2003). This involves giving the control animal the same amount of food that has been eaten by the treatment animal on a daily basis. It ensures that the effects seen in the treatment group are due to the drug alone and not due to any nutritional deficits. This method is widely accepted as a nutritional control. However, there are problems with this type of food restriction in that the dams may consume their food allocations within the first few hours of feeding. Hence, they are then on 'deprivation schedule'

and this may interfere with circadian rhythms and alter hormonal stress response patterns (Mattsson, 1994). In order to overcome this the food should be placed in with the animal during the dark phase of each day as this is the time when the animal will eat the most and so this 'deprivation schedule' could be alleviated.

1.2.2 Offspring experimental variables

The next section will look at other experimental parameters regarding the offspring to see if there is consistency within the animal model. Many experimental factors regarding the offspring need to be considered including litter standardisation and cross fostering, behavioural assessments and developmental parameters (Figure 1.5).

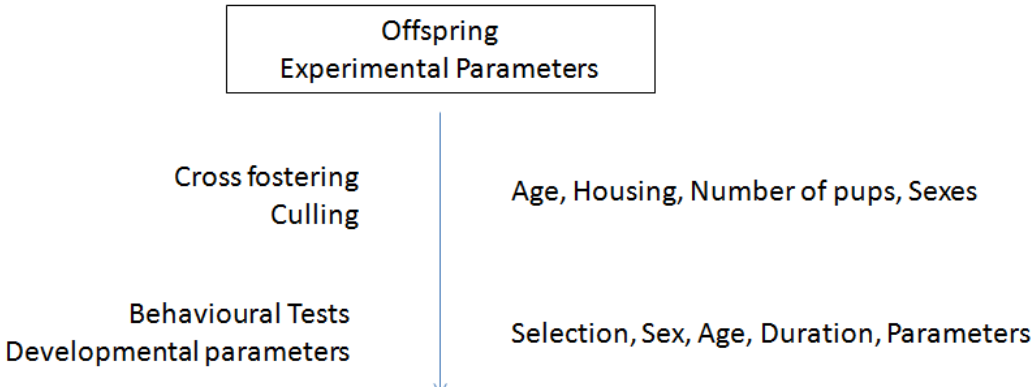

Figure 1.5: Offspring experimental variables. Chart depicting the offspring experimental variables that exist for ATS drug studies.

Litter standardisation and cross fostering

After birth, litters can be standardised to ensure equal numbers of pups across all litters and also equal number of males and females in all litters. For developmental studies standardisation is optional. However, standardisation is beneficial as it ensures that all mothers have to care for and feed the same number of pups and

therefore each pup is receiving the same care from the mother especially concerning feeding (Agnish and Keller, 1997). A very small litter undoubtedly would gain more weight in comparison to a very large litter as there is less competition for feeding (Chahoud and Paumgartten, 2009). If the pups are being culled, it is recommended that it is performed on PND 4 and that the litter is culled to 8 or 10 pups with the same number of males and females where possible (U.S. Environmental Protection Agency, 1998, OECD Organisation for Economic Co-operation and Development, 2007, Agnish and Keller, 1997). This method has been incorporated by many ATS studies (Vorhees *et al.*, 2009, Williams *et al.*, 2006, Heuland *et al.*, 2010, Smith and Chen, 2009) but there has been a variation in the time at which litters have been standardised. Although the recommended PND is 4, most studies have culled litters on PND 1 (Schaefer *et al.*, 2006, Skelton *et al.*, 2009, Flores *et al.*, 2011) and other PNDs used for standardisation include PND 0 (Gomes-da-Silva *et al.*, 2002, Heuland *et al.*, 2010), PND 2 (Laviola *et al.*, 2001), PND 3 (Barenys *et al.*, 2010, Acuff-Smith *et al.*, 1996) and PND 7 (Martin, 1975) (See Tables 1.8.1, 1.8.2 and 1.8.3). Martin *et al.* (1976) states that by waiting to cull the pups a few days after birth then the 'healthiest' offspring will remain for the experiment as the pups that are most affected by MA will die or be cannibalized soon after birth. Pup selection should be completely random (randomisation technique recommended) and unbiased. There should be no bias in choosing the pups to continue into testing especially in trying to eliminate underweight pups, sick pups or runts (Palmer, 1986, Agnish and Keller, 1997). Neonatal death can still occur in the second and third postnatal weeks (Chahoud and Paumgartten, 2009) and so litter sizes may still change after standardisation.

Cross fostering is also an important factor to consider. Cross fostering is generally employed in order to eliminate any possibility that altered maternal behaviour may affect the development of the pups. Therefore, regardless of prenatal treatment, all pups will be exposed to the same postnatal care from the mother. It has been previously shown that MA pups cross fostered to a control mother can overcome the impairing effect of prenatal MA exposure and have better performance in sensorimotor tests compared to MA pups reared by MA mothers (Pometlova *et al.*, 2009). Many ATS studies have used this technique (Chen *et al.*, 2010, Tan, 2003) (Tables 1.8.1, 1.8.2 and 1.8.3) however, with regards drug exposure, and in particular ATS exposure, there are risks associated with this technique. We cannot be sure that an ATS exposed mother that is fostered to control pups does not have active drugs present in which could be passed onto the control pups via breast milk or urinary and faecal excretions, especially since cross fostering is most frequently done on PND 1 with 73% of ATS studies doing this (See Tables 1.8.1, 1.8.2 and 1.8.3). In adult rats it has been shown that when given orally 10% of MA appears unchanged in the urine (Caldwell *et al.*, 1972). Clinical results have also shown that MA is present at birth in meconium samples of new-borns of MA-abusing mothers (Arria *et al.*, 2006). Therefore, as argued by McDonnell-Dowling *et al.* (2014), there is also a possibility that the fostered ATS pups may still have active drugs present after birth and this could indirectly expose the control mother to the drug via urinary and faecal excretions. Although there is no data to support this, we cannot be sure that this is not a possibility and this is something that may warrant further investigation. If one is to replicate the clinical situation then the pups should be left with their biological mothers and reared by their biological mothers. In the clinical scenario, altered behaviour of the mother after birth, caused by MA use, may also

contribute to deficits in development of their children and so we should aim to keep the animal model as close as possible to what occurs in humans. In order to see if maternal behaviour is altered then this can be monitored postnatally by looking at behaviours such as licking, grooming and feeding of the pups and comparing to a control group. An observation time of 10 min on each PND however, may not be enough to accurately depict the overall maternal care towards fostered pups. Maternal behaviours should ideally be repeated and performed across the postnatal period after cross fostering occurs. A prolonged observation time is also preferred to get a comprehensive idea of how the mother is behaving towards the fostered young. Slamberova *et al.* (2007) looked at maternal behaviour by using the observational test from PND 1-22 and the retrieval test from PND 1-12. This study design is ideal as one can obtain a large amount of information while keeping the disruption to the mother and litter at a minimum.

Cross-fostering	Culled	PND	Culled to	# M+F	No. of papers
N	N	-	-	-	15/69
N	Y	0	8	4M+4F	1/69
N	Y	1	8	4M+4F	8/69
N	Y	1	8	6M+2F	3/69
N	Y	3	8	4M+4F	1/69
N	Y	4	8	8M	2/69
Y	Y	?	8	NS	1/69
N	Y	7	8	NS	1/69
Y	Y	0	9	5M+4F	1/69
N	Y	0	10	5M+5F	1/69
N	Y	1	10	5M+5F	4/69
Y	Y	1	10	5M+5F	8/69
Y	Y	1	10	NS	1/69
Y	Y	2	10	5M+5F	1/69
N	Y	?	10	10M	1/69
Y	Y	0	12	6M+6F	1/69
Y	Y	1	12	NS	1/69
Y	Y	1	12	6M+6F	13/69
N	Y	1	12	6M+6F	1/69
Y	Y	1	?	NS	1/69
Y	Y	2	12	6M+6F	1/69

Table 1.8.1: Cross fostering and standardisation in MA studies. Use of cross fostering and standardisation employed in MA drug studies. n=69 MA papers. Y, Yes; N, No; M, Male; F, Female; NS, Not Specified.

Cross-fostering	Culled	PND	Culled to	# M+F	No. of papers
N	N	-	-	-	6/20
N	Y	1	8	4M+4F	4/20
Y	Y	1	8	4M+4F	1/20
Y	Y	1	8	8M	1/20
N	Y	1	8	6M+6F	1/20
N	Y	1	10	NS	1/20
N	Y	3	10	10M	1/20
N	Y	0	10	NS	1/20
N	Y	1	8/10	4M+4F/ 5M+5F	4/20

Table 1.8.2: Cross fostering and standardisation in MDMA studies. Use of cross fostering and standardisation employed in MDMA drug studies. n=20 MDMA papers. Y, Yes; N, No; M, Male; F, Female; NS, Not Specified.

Cross-fostering	Culled	PND	Culled to	# M+F	No. of papers
N	N	-	-	-	7/19
Y	N	-	-	-	1/19
N	Y	2	6	6M	1/19
N	Y	1	8	4M+4F	5/19
N	Y	0	8	NS	1/19
Y	Y	0	8	4M+4F	1/19
Y	Y	1	8/10	4M+4F/ 5M+5F	1/19
N	Y	1	10	10M	1/19
N	Y	1	?	NS	1/19

Table 1.8.3: Cross fostering and standardisation in AMP studies. Use of cross fostering and standardisation employed in AMP drug studies. n=19 AMP papers. Y, Yes; N, No; M, Male; F, Female; NS, Not Specified.

Behavioural Tests and Developmental Parameters

The tests chosen to examine the development of offspring that have been exposed to ATS drugs during pregnancy and/or lactation are very important and are dependent on the specific abnormalities that are to be assessed. Pup development can be subdivided into somatic and behavioural and in order to get a clear picture of the offspring development one should endeavour to look at both of these aspects. Somatic development refers to the physical development of the offspring and the date at which they achieve this developmental parameter. Most studies will look at body weights and weight gain for this (Chen *et al.*, 2010, Barenys *et al.*, 2010, Smith and Chen, 2010) however, other parameters that can be assessed including pinna unfolding, eye opening, incisor eruption, fur appearance, body length and ano-genital distance (Heuland *et al.*, 2010, Cho *et al.*, 1991). Again, this is measured at the day at which these parameters occur or appear. Behavioural development uses various tests to evaluate how the offspring perform. Tables 1.9.1 and I.9.2 show a variety of behavioural tests that have previously been employed during the neonatal, adolescent and adult periods and what these tests are used to evaluate. For neonatal behavioural testing the tests employed usually involve measuring the pups strength, co-ordination and balance whereas adolescent and adult behavioural tests can be divided into locomotor activity, anxiety, depression, pain, memory and learning.

Behavioural Test used	Measures/Function	Reference
Cliff Avoidance Test	Integration of exteroceptive input and locomotor output	(Fan *et al.*, 2005)
Surface Righting Reflex	Development of body righting mechanisms and the maturation of vestibular function	(Khan *et al.*, 2004) (Mesquita *et al.*, 2007)
Negative Geotaxis	Dynamic tests of sensorimotor development	(Mesquita *et al.*, 2007)
Air Righting Reflex	Development of body righting mechanisms and the maturation of vestibular function	(Khan *et al.*, 2004) (Mesquita *et al.*, 2007)
Bar-Holding Test	Strength as well as vestibular function and sensor -motor coordination	(Sousa *et al.*, 2006) (Mesquita *et al.*, 2007)
Tail Pull	Strength	(Hruba *et al.*, 2009b)
Rotarod	Coordinated motor and balance skills	(Khan *et al.*, 2004)

Table 1.9.1: Behavioural tests used in the neonatal period. Various behavioural tests used to assess development in neonate stages after ATS exposure.

Behavioural Test used	Measures/Function	Reference
Learning and memory		
Barnes maze	Spatial learning	(Vorhees, 1997)
Morris Water Maze	Spatial learning	(Sousa *et al.*, 2006)
Conditioned avoidance response	Categorical learning	(Sousa *et al.*, 2006)
Novel object recognition	Recognition memory	(Sousa *et al.*, 2006)
Passive avoidance learning	Categorical learning and memory	(Sousa *et al.*, 2006)
Cincinnati Water Maze	Learning and memory	(Vorhees, 1997)
Pain		
Formalin test	Nociceptive sensitivity	(Chen *et al.*, 2010)
Hot Plate	Sensitivity to a thermal stimulus	(Miyakawa *et al.*, 2001)
Anxiety		
Light:dark box	Anxiety-related behaviours	(Sidor *et al.*, 2010)
Open field	Anxiety-related behaviours	(Sousa *et al.*, 2006)
Elevated Plus Maze	Anxiety-related behaviours	(Sidor *et al.*, 2010)
Marble burying	Anxiety-related behaviours	(Skelton *et al.*, 2009)
Locomotor		
Straight Channel	Swimming speed and ability	(Acuff-Smith *et al.*, 1996)
Home cage activity	Home cage locomotor activity	(Sousa *et al.*, 2006)
Open field	Locomotor activity	(Sousa *et al.*, 2006)
Rotarod	Coordinated motor and balance skills	(Khan *et al.*, 2004)
Depression		
Forced swim	Depressive-like behaviours	(Porsolt *et al.*, 1978)
Motivation		
Conditioned place preference	Motivational effects of objects or experiences	(Slamberova *et al.*, 2011b)
Running wheel activity	Measure of voluntary exercise	(Thompson *et al.*, 2009b)
Responding for sucrose	Food-motivated behaviour	(Thompson *et al.*, 2009b)

Behavioural Test used	Measures/Function	Reference
Other		
Crossed extensor reflex	Withdrawal reflex measurement	(Heuland *et al.*, 2010)
Acoustic startle response	Reaction to acoustic stimuli	(Mesquita *et al.*, 2007)
Prepulse inhibition	Startle reflex measurement	(Miyakawa *et al.*, 2001)

Table 1.9.2: Behavioural tests used in the adolescent and adult periods. Various behavioural tests used to assess development adolescent and adult stages after ATS exposure.

For each of these tests one must take into account how these tests are performed and considerations including the age of rats at testing, the duration of the testing period, the length of tests and sex of pups tested. These aspects are generally similar between studies however, some variation in the literature exists. The rotarod test is a test of coordinated motor and balance skills (Khan *et al.*, 2004) which has been used in both neonatal periods and adulthood and involves placing the animal on a rod or cylinder that rotates. The objective of this test is to see how long the animal can stay on before falling off or without falling off. The duration of the test in the neonatal period is most commonly set at 120 sec (Pometlova *et al.*, 2009, Hruba *et al.*, 2009b, Slamberova *et al.*, 2007). This means that whether the pup has fallen off the apparatus or not the test is terminated after 120 sec. However, some studies have performed this test in the neonatal period for 5 min with increasing speed (Barenys *et al.*, 2010) which is an incredibly long time for a young pup. The amount of trials that are performed for tests by each animal is another aspect that can vary. For instance, with the rotarod used for rat offspring, the rat may perform the trial three times with an inter-trial interval of 1 min (Pometlova *et al.*, 2009) or they may need to repeat the task until it is successfully accomplished with a maximum of 10 trials

(Slamberova *et al.*, 2007). This lack of standardisation between labs for simple behavioural assessments means that one animal may be performing the behavioural test once and another may be performing it up to 10 times. As each animal may perform a different version of the test it makes it difficult to interpret the results obtained. The use of the three trials regardless of performance is useful in that it controls for the amount of times that an animal is exposed to the test, the amount of time between each test and obtaining an average value on how each animal performed between the three trials. Then a deficit in coordinated motor and balance skills would be easily apparent and could be correlated directly to the drug treatment and not to the test parameters.

The age of rats at the time of testing is important. Each test looks at a different developmental parameter and so at a different developmental time point. The surface righting reflex looks at the development of body righting mechanisms and the maturation of vestibular function (Mesquita *et al.*, 2007, Khan *et al.*, 2004). The age of pups at the time of testing for this has varied among studies with some looking at an early time point such as PND 4 (Cho *et al.*, 1991) and some looking at later time points in the neonatal period such as PND 12 (Hruba *et al.*, 2009b). The earlier time points are more suited for this test as this is when development is occurring in the offspring and therefore one can determine if there is in fact a developmental delay in this parameter. The timing and execution of these common neurodevelopmental parameters needs to be standardised so that there is one common version of each test rather than multiple variations of each parameter.

Finally, for each parameter it is vital that both male and female pups are tested. Many studies have claimed that they have found no sex difference between males and females on common neurodevelopment parameters (Pometlova *et al.*, 2009, Hruba *et al.*, 2009b, Grace *et al.*, 2010) and so it is quite common for studies to only look at male pups (Flores *et al.*, 2011, Thompson *et al.*, 2009b, Slamberova *et al.*, 2011b). However, many studies have shown that there is indeed a sex difference (McDonnell-Dowling *et al.*, 2014, Vorhees *et al.*, 2009, Vorhees *et al.*, 2008, Yamamotova *et al.*, 2011, Slamberova *et al.*, 2010b) and that female pups may even be more susceptible to neurotoxicity from an ATS drug (Rambousek *et al.*, 2014). Results for each sex can vary between studies and in adulthood it is important to take the oestrus phase into account as this can alter the female's behaviour. As both sexes are available from birth then it makes sense to study the effects of these drugs in both males and females.

Upon examining these studies, it is again clear that there is also a large diversity among these studies regarding the offspring experimental variables. The next section will look at the recommendations for both the maternal and offspring experimental parameters that should be employed in an ATS exposure study during pregnancy and/or lactation.

1.3 Conclusions

The previous sections have focused on the range of experimental parameters that exist in an animal model of ATS exposure *in utero* and during lactation. From looking at the various study designs that exist it is clear that there is a large diversity and little consistency among these studies and so the interpretation of these results

may not be as clinically relevant as previously thought. Our recommendations for the maternal experimental parameters in an ATS study are outlined in Figure 1.6 and this acts as a guideline for conducting these studies. For animals being used it is important to choose the most appropriate species and strain relevant for each study, to use naïve females that have not been previously mated and to also ensure that the animal is of a reproductive age and that all details regarding weight and age of the animal are disclosed. Here we recommend that in a rat model (either Sprague-Dawley or Wistar) females are 3 months old and weighing between 250 and 300g. For the mating procedures it is important to use a reliable method to confirm that mating has taken place. Here we recommend that mating should occur overnight between one male and one female and most importantly that confirmation of copulation is obtained using a vaginal smear and that this day is considered to be day 0 of pregnancy (GD 0). For ATS studies, the dose, route of administration, duration and frequency of exposure are all pivotal to the study design (Figure 1.6) and all of which should reflect the clinical situation. The recommended preclinical study investigating the effects of ATS drug exposure during pregnancy and/or lactation in a rat would involve using a dose of 2.5 to 4 mg/kg giving daily via oral gavage. Multiple exposure durations should be investigated that look at each possible clinical pattern of exposure and the drug should be administered directly to the mother. Control mothers for this experiment should be given the vehicle at the same volume and at the same time as the treated mothers. Throughout the gestation and lactation periods similar outcome effects should be monitored that are seen clinically such as body weight, food consumption, water consumption and maternal behaviour. By keeping these parameters as clinically relevant as possible it will also allow us to be

more confident in the results obtained and confident that the human situation is being replicated as closely as possible.

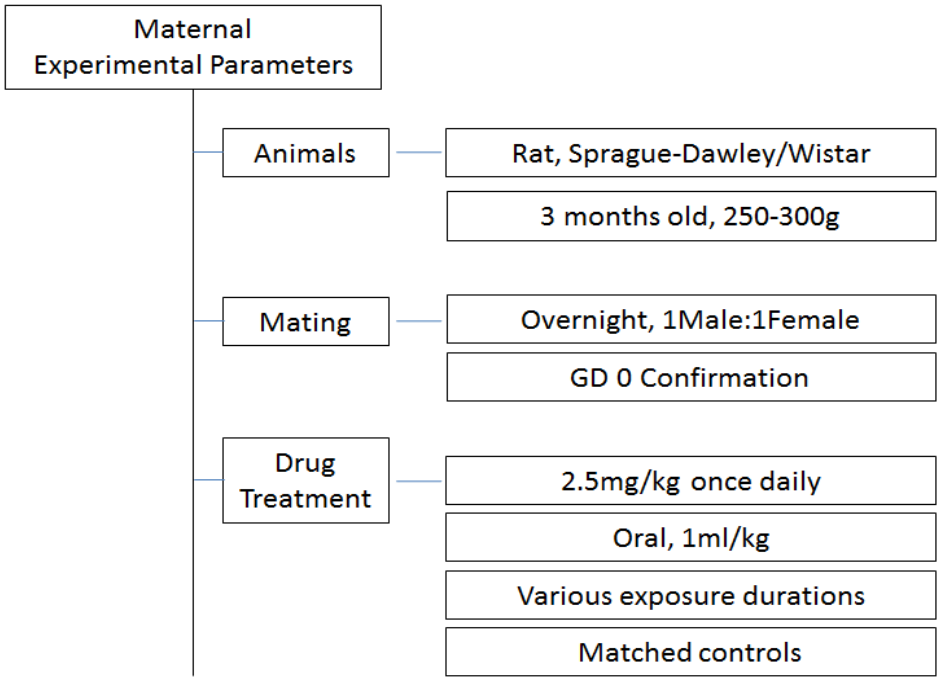

Figure 1.6: Recommended maternal experimental variables. Chart depicting the ideal maternal experimental variables for an ATS drug study.

Our recommendations for pup standardisation and cross fostering is that cross fostering is not performed (but maternal behaviour is recorded) and that the pups remain with their birth mothers but that standardisation is performed on PND 1 (10-12 pups) and that the same number of males and females are kept where possible. Male and females pups should both be kept and both should be tested throughout the entire neonatal period, looking at as many parameters as possible. The offspring experimental variables regarding testing depend solely on what is being assessed in each study. Therefore, the recommendations are not aimed at which tests are better but more so that the tests performed and the parameters assessed are more consistent

both within a single study and between studies and laboratories. The standardisation of these simple tests mean that results will be more comparable between papers and that results generated will give us a true understanding of what can and may be happening in the clinical scenario.

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Chapter 1:

Introduction II:

The role of oxidative stress in methamphetamine-induced toxicity and sources of variation in the design of animal studies

McDonnell-Dowling, K. & Kelly, J. P. 2016. The role of oxidative stress in methamphetamine-induced toxicity and sources of variation in the design of animal studies. *Current Neuropharmacology*, Under Review.

Abstract

The prevalence of MA use has increased in recent years. In order to assess how this drug produces its effects, both clinical and preclinical studies have recently begun to focus on oxidative stress as an important biochemical mechanism in mediating these effects. The purpose of this review is to illustrate the variation in the design of preclinical studies investigating MA exposure on oxidative stress parameters in animal models. The experimental variables investigated and summarised include MA drug treatment, measurements of oxidative stress and antioxidant treatments that ameliorate the harmful effects of MA. These preclinical studies differ greatly in their experimental design with respect to the dose of MA (ranging between 0.25 to 20 mg/kg), the dosing regime (acute, binge or chronic), the time of measurement of oxidative stress (0.5 h to 2 wks after last MA administration), the antioxidant system targeted and finally the use of antioxidants including the route of administration (ip or po), the frequency of exposure and the time of exposure (preventative or therapeutic). The findings in this paper suggest that there is a large diversity among these studies and so the interpretation of these results is challenging. For this reason, the development of guidelines and how best to assess oxidative stress in animal models may be beneficial. The use of these simple recommendations mean that results will be more comparable between laboratories and that future results generated will give us a greater understanding of the contribution of this important biochemical mechanism and its implications for the clinical scenario.

1.4 Introduction II

1.4.1 Methamphetamine and Theories of Toxicity

With the increasing use of MA worldwide there has been a growing awareness of the harmful effects of MA exposure, and explorations of the biochemical mechanisms by which such effects are mediated. Its mechanisms of toxicity have been studied extensively and it appears that MA can exert its toxic effect in many ways. Although attempts have been made to explain MA-induced toxicity by its links to excitotoxicity (excessive glutamate release), mitochondrial dysfunction (Quinton and Yamamoto, 2006), blood brain barrier dysfunction, inflammation and DNA damage (Krasnova and Cadet, 2009), oxidative stress has shown to be a promising lead in explaining, at a cellular level, the harmful effects of MA abuse. Figure 1.7 highlights the cascade of events that occur after MA exposure.

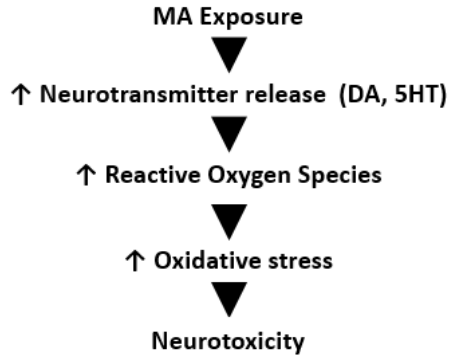

Figure 1.7: MA cascade of events. The implications of MA exposure. DA; Dopamine, 5-HT; Serotonin. (Adapted from Halpin *et al.* (2014)).

1.4.2 Oxidative Stress

MA is an amphetamine-type stimulant that crosses the blood–brain barrier easily and stimulates the CNS by acting as a sympathomimetic drug (Kraemer and Maurer, 2002). MA is known to increase the synaptic availability of dopamine (DA) and serotonin (5HT) (Yamamoto and Raudensky, 2008). These are acute effects of MA

however and it is thought that chronic exposure to MA can result in neurotoxicity and long-lasting damage to the dopaminergic axon terminals (Ricaurte *et al.*, 1982). Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defences which can result in damage (Betteridge, 2000). After MA administration the increased DA undergoes auto-oxidization to toxic products known as ROS including hydrogen peroxide (H_2O_2), hydroxyl radicals (OH·) and superoxide radicals ($O_2\cdot^{-}$) (Figure 1.8). If these are not detoxified by antioxidants or antioxidative enzymes, they may damage proteins, lipids, DNA and RNA (Wells *et al.*, 2005).

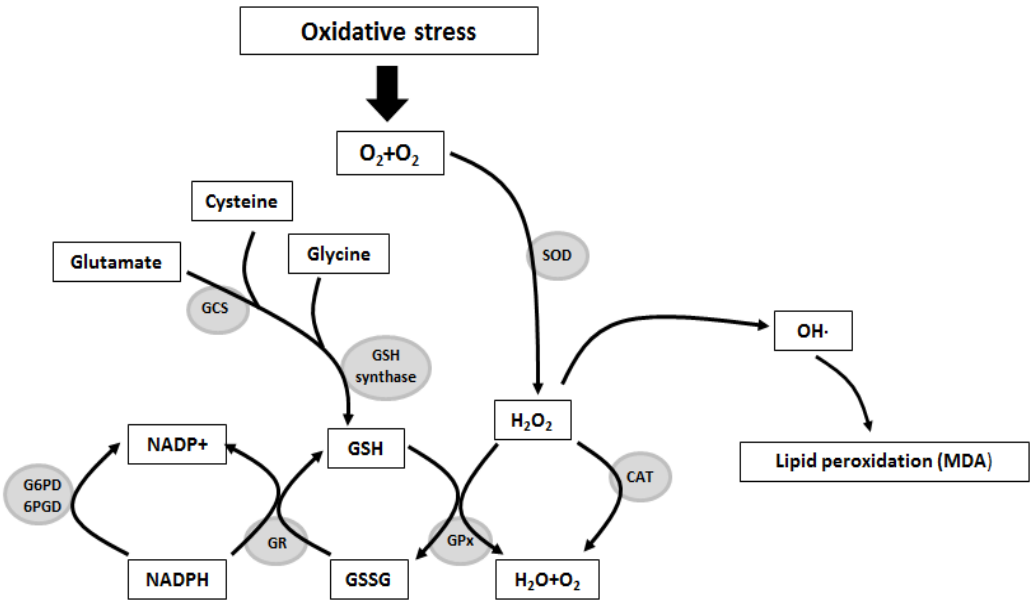

Figure 1.8: Oxidative stress pathway. The implications of MA exposure on oxidative stress. SOD; Super Oxide Dismutase, MDA; Malondialdehyde, CAT; Catalase, GSH; Glutathione, GCS; Glutamylcysteine synthetase, GSSG; Oxidised glutathione, GR; Glutathione reductase, GPx; Glutathione peroxidase, G6PD; Glucose-6-phosphate dehydrogenase, 6PGD; 6-phosphogluconate dehydrogenase, NADPH; Nicotinamide Adenine Dinucleotide Phosphate-Oxidase, OH; Hydroxyl

Radical, H_2O_2; Hydrogen Peroxide. (Adapted from Halpin *et al.* (2014), Guo and Chen (2012), Isagenix International (2015)).

The antioxidant defence system acts as a scavenging system to detoxify these free radicals and prevent or minimise cellular damage. Catalase (CAT), glutathione peroxidase (GPx) and Super-Oxide Dismutase (SOD) are scavenger enzymes. However when the system is overloaded these enzymes can be decreased or inactivated by oxidative stress which makes the task of defending against ROS quite challenging (Krasnova and Cadet, 2009, Cadet and Brannock, 1998).

1.4.3 Neurotoxicity

When the antioxidant defence system fails or is overloaded, the ROS generated can lead to cellular damage by acting on nucleic acids, proteins and phospholipids (Ares-Santos *et al.*, 2013). Therefore this increased concentration of DA and changes in DA metabolism after MA exposure can switch on the oxidative stress cascade and consequently can lead to the degeneration of dopaminergic terminals (Ares-Santos *et al.*, 2013). The literature to date has shown that MA-induced neurotoxicity is dependent on dopamine levels and because of this the striatum and nucleus accumbens are the brain regions that have been studied most extensively. These regions have the most robust dopaminergic projections and so are most susceptible to MA-induced oxidative damage (Johnson *et al.*, 2015).

1.4.4 The preclinical picture

The first implications that oxidative stress plays a role in MA-induced neurotoxicity date back to 30 years ago, when it was discovered that the antioxidants vitamin E (α-

tocopherol) and ascorbic acid attenuated the depletions of DA and 5HT seen in the striatum after MA exposure (Wagner *et al.*, 1985, De Vito and Wagner, 1989). Since then, there has been much interest in this topic, using both animals and humans. Although there have been significant results to date in animal studies, there has been considerable variation between studies and how oxidative stress is measured. The purpose and aim of each study varies but when investigating the effects of MA on oxidative stress it is still unclear which treatment regime, dose, sacrifice time, brain region etc. yields the most significant changes in oxidative stress. Due to this wide range of experimental protocols that exist, the interpretation of these different results is challenging. For this reason, the development of guidelines and how best to assess oxidative stress in animal models may be beneficial. Simple recommendations like these mean that results will be more comparable between papers and that future results generated will give us a greater understanding of the contribution of oxidative stress and its implications for the clinical scenario

1.4.5 Aims

The purpose of this review is to examine the evidence implicating oxidative stress with MA exposure in human and animal models and to also propose an optimum protocol for assessing oxidative stress in laboratory animal models exposed to MA.

1.4.6 Review methods

The search terms ‘Methamphetamine AND Oxidative Stress’ were entered into PubMed search engine. Between 1990 and 2014, 220 articles were published in this area (Figure 1.9). Among these articles 41 studies were relevant in that they involved MA exposure and oxidative stress.

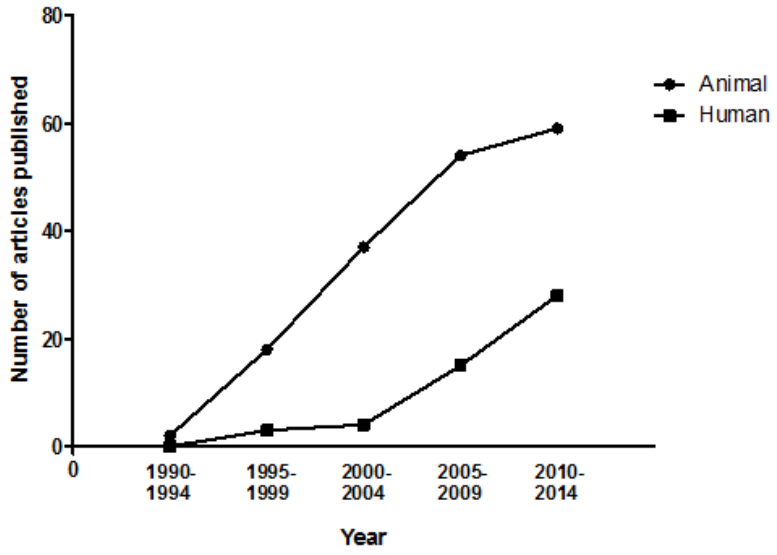

Figure 1.9: Papers published. Total number of articles returned when 'Methamphetamine AND Oxidative Stress' was entered into PubMed search engine.

1.5 How to measure oxidative stress?

In the literature, the methods used for exploring the effects of MA use on oxidative stress in animals vary substantially. By separately focusing on each stage of the experimental design the variables can be categorised accordingly (Figure 1.10).

3) MA drug treatment (Dose, regime, length of exposure, route of administration).
4) Measuring oxidative stress (Time point following administration, brain region(s), parameter/targets to use).
5) Antioxidant treatments (Type of antioxidant, regime, time of exposure, route of administration).

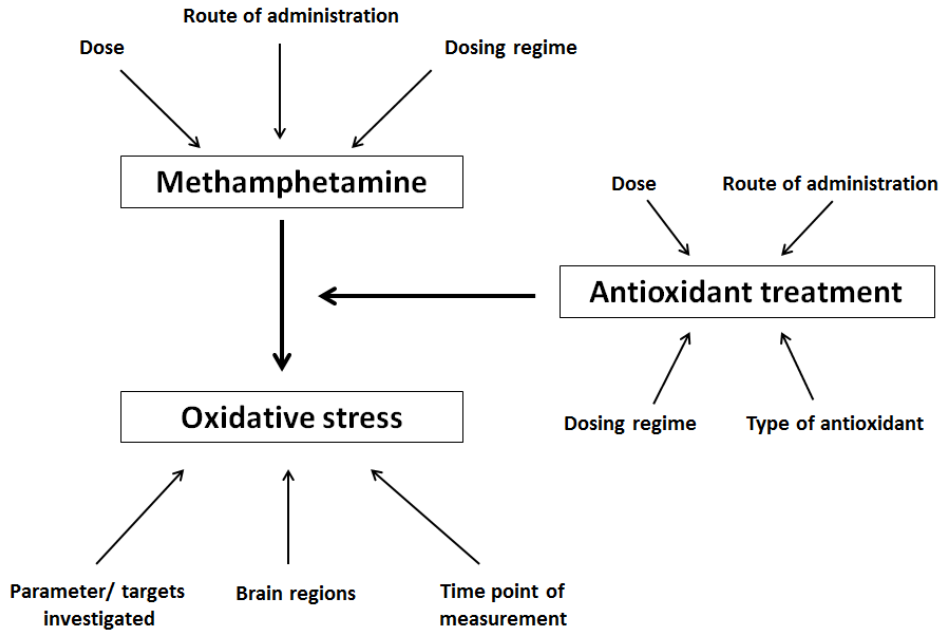

Figure 1.10: Experimental variables. Chart depicting the experimental variables that exist in MA oxidative stress studies.

1.5.1 MA drug treatment

Dose

The first documented preclinical studies examining the role of oxidative stress and MA use used doses of 5 and 10 mg/kg (Hirata *et al.*, 1995), but in the intervening years a wide range of MA doses have been employed**.** We previously classified these MA doses into neurotoxic, toxic and pharmacological (McDonnell-Dowling and Kelly, 2015) and Table 1.10 classifies the MA doses used from these studies accordingly. Although some studies have looked at lower MA doses (pharmacological and toxic), most of the doses employed to date have fallen into the neurotoxic class range (i.e. >5 mg/kg).

Dose Classification	Comments	Dose Range	% of preclinical papers
Neurotoxic	Resemble extremely high toxic doses	≥5 mg/kg	64
Toxic	Resemble high pharmacological doses	2-4 mg/kg	12
Pharmacological	Resemble doses used in pharmacological studies	0.25-2 mg/kg	24

Table 1.10: Classification of previously used preclinical doses for MA. The range of doses used in MA oxidative stress preclinical studies (Adapted from McDonnell-Dowling and Kelly (2015).

The minimum dose of MA used previously has been 0.25 mg/kg (da-Rosa *et al.*, 2012a, da-Rosa *et al.*, 2012b). When this dose was employed for 7 days, the authors reported increased carbonyl group formation and lipid damage in all brain regions examined, namely the prefrontal cortex, amygdala, hippocampus and striatum (da-Rosa *et al.*, 2012b), suggesting marked effects on oxidative stress even following a relatively low dose of MA. At the other extreme, the maximum dose of MA has been 80 mg/kg as a "binge" dose (four 20 mg/kg injections in one day) or 20 mg/kg as a chronic dose (20 mg/kg once daily for 10 days) (Moszczynska *et al.*, 1998). The total glutathione (GSH) concentration was reduced in the striatum of the MA binge group and the glucose-6-phosphate dehydrogenase (G6PD) activity was reduced in the frontal cortex of the MA chronic group. Although these changes suggest that oxidative stress may be involved in MA-induced neurotoxicity, these changes were only small (slight reductions) and so compared to a low dose of 0.25 mg.kg, there is no greater effect with these higher doses. The most common MA dose previously

employed has been 10 mg/kg with 50% of the MA and oxidative stress studies using this dose (Mori *et al.*, 2007, Thrash-Williams *et al.*, 2013, Gluck *et al.*, 2001, Yamamoto and Zhu, 1998). At this dose studies have reported many alterations in oxidative stress parameters (Table 1.11).

Effect	Parameters							
↑	ROS	SOD activity	MDA	GPx levels	Protein carbonyls	CAT activity	GSSG content	Uric acid content
↓	GSH levels	GPx levels						

Table 1.11: Effects of 10 mg/kg MA on oxidative stress parameters. The range of oxidative stress parameters altered after MA preclinical studies. (Koriem *et al.*, 2013, Banerjee *et al.*, 2010, Toborek *et al.*, 2013, Zhang *et al.*, 2012, Thrash-Williams *et al.*, 2013, Harold *et al.*, 2000, Flora *et al.*, 2002, Gluck *et al.*, 2001, Yamamoto and Zhu, 1998)

The pharmacological doses (0.25-2 mg/kg) and low toxic doses (2-3 mg/kg) of MA (Table 1.10) are the doses that equate to those most commonly abused in the clinical scenario (McDonnell-Dowling and Kelly, 2015). Therefore it is pertinent that such doses have shown to yield the greatest results for oxidative stress as it allows for comparison of these preclinical studies to what is happening in the human situation.

Dosing regime

The frequency of MA dosing or the amount of MA injections given on each day varies between each study. The most common frequencies are one dose per day (da-Rosa *et al.*, 2012a, Mori *et al.*, 2007, Acikgoz *et al.*, 2000) or “binge” dosing with

four doses per day (with 2 h intervals separating the doses) (Sinchai *et al.*, 2011, Hirata *et al.*, 1995, Fukami *et al.*, 2004, Yamamoto and Zhu, 1998) (Table 1.12).

No. injections/day	Time interval between injections	% of papers
1	N/A	30
2	2 h apart	4
2	10 h apart	4
2	12 h apart	4
3	2 h apart	4
3	3 h apart	4
4	2 h apart	35
4	5 h apart	4
5	2 h apart	9

Table 1.12: Dosing routines. The range of injection routines used in MA oxidative stress preclinical studies.

The use of multiple injections in one day is generally used as a neurotoxic regime. Rather than trying to achieve a clinical pattern of MA use this method is overloading the protective mechanisms by using a continuous assault of the drug. This method can achieve a higher daily dose without running the risk of giving the animal an overdose or reaching the LD_{50}. Many variations exist for this regime including 2 injections in a day (2 h, 10 h or 12 h apart) (Thrash-Williams *et al.*, 2013, Bu *et al.*, 2013, Zhang *et al.*, 2013), 3 injections in a day (2 h or 3 h apart) (Banerjee *et al.*, 2010, Chen *et al.*, 2012), 4 injections in a day (2 h or 5 h apart) (Sinchai *et al.*, 2011, Moszczynska *et al.*, 1998) and 5 injections in a day (2 h apart) (Kita *et al.*, 2000). However, as seen in the previous section, preclinical studies have found that oxidative stress changes following MA administration can be detected after small

doses (0.25 – 0.5 mg/kg, one injection) and therefore the use of a neurotoxic regime may not be causing an increased state of oxidative stress. However, the number of days in which the animal receives the drug can also vary among studies. One day of administration is generally the most common dosing routine and this is combined with multiple injections in a single day. When only one injection is given in a day this is most commonly given for multiple days. Therefore the main strategies that have been previously employed are focusing on either chronic/long term dosing or acute/binge dosing. Chronic dosing (multiple days of dosing) ranges between 4 and 14 days of dosing and figure 1.11 shows all the routines previously used.

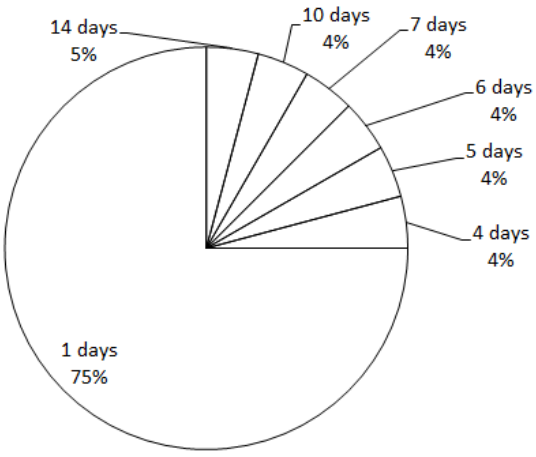

Figure 1.11: Drug administration routines. The range of drug administration routines used in MA oxidative stress preclinical studies.

So how do acute, binge, multiple days or chronic dosing compare regarding their effects on oxidative stress? Firstly, looking at acute dosing, da-Rosa *et al.* (2012a) showed that acute MA increased carbonyl group formation and Thiobarbituric acid reactive substances (TBARS) generation in the prefrontal cortex, amygdala, hippocampus and striatum at doses 0.5, 1 and 2 mg/kg (by over 100%). A study by

Acikgoz *et al.* (2000) found that when given as a single injection for one day at a dose of 5 mg/kg, MA had no effect on TBARS levels, GPx activity or SOD activity in the striatum or prefrontal cortex. However, when administered acutely at 15 mg/kg there was an increase in TBARS levels in both the striatum (by over 150%) and prefrontal cortex (by over 300%) and when given at 10 mg/kg there was an increase in SOD activity in the prefrontal cortex (by over 40%). An acute MA dose of 10 mg/kg increased TBARS levels in the corpus striatum, hippocampus and frontal cortex, increased GSH content in the striatum and frontal cortex, and increased GPx activity in the striatum and frontal cortex (by 30%) (Flora *et al.*, 2002). A binge dose could be considered a high MA dose that is given several times in a single day. For example Moszczynska *et al.* (1998) administered MA at 20 mg/kg and this was given 4 times in one day at 5 h intervals. In this study, the total GSH concentration was reduced in the striatum of the MA binge group although this reduction was only small (-17%) compared to a 47% increase in total GSH levels after acute MA at 10 mg/kg in the aforementioned study by Flora *et al.* (2002). However, in a similar dosing regimen of MA, at only 7.5 mg/kg given 4 times in one day at 2 h intervals, showed significant increases in malondialdehyde (MDA) formation, 4-hydroxynonenal (4-HNE) expression, ROS formation and protein carbonyl expression (Shin *et al.*, 2011). The same authors showed that after MA, at 8 mg/kg given 4 times in one day at 2 h intervals, MDA and protein carbonyl content were also increased in the striatum (Shin *et al.*, 2012). There are limited preclinical studies that have examined multiple dosing days or chronic dosing (Table 1.13). As stated earlier, da-Rosa *et al.* (2012b) showed that at 0.25 mg/kg of MA for 7 days increased carbonyl group formation and lipid damage in all brain regions examined. When MA was given chronically at this same dose for 14 consecutive days (one

daily injection) the authors also found increased carbonyl group formation and TBARS generation in the prefrontal cortex, amygdala, hippocampus and striatum (da-Rosa *et al.*, 2012a). Pang *et al.* (2013) showed that 6 consecutive daily injections of MA at 2.5 mg/kg resulted in higher GSH contents in the striatum, decreased glutamylcysteine synthetase (GCS) activities in striatum and frontal cortex, decreased activity of glutathione reductase (GR) in the thalamus and increased activity of GR in the striatum and glutaredoxin (Glrx) activity was upregulated in the striatum. At a higher dose of 20 mg/kg for 10 consecutive days (one daily injection) MA only reduced the G6PD activity in the frontal cortex but had no effects on levels of GSH or activities of the other glutathione-related enzymes (GPx, GR, g-GTP (g-glutamyltranspeptidase)) in any of the brain regions (cerebellum, frontal cortex, striatum) (Moszczynska *et al.*, 1998). It is interesting to see differences in oxidative stress results after different dosing regimens as although clinically chronic, long-term use is most common, there are a diversity of individual abuse patterns (National Survey on Drug Use and Health, 2005). After looking at all the variations of how MA is given to the animals it is clear that the dose of drug given must be the most important parameter when assessing oxidative stress. The differences in results between studies can be mostly explained by the dose of drug rather than the dosing regime itself. Whether given short term or long term oxidative stress can be detected using various parameters; however when given at low doses (pharmacological doses, Table 1.10) the results yielded seem to be more significant and of a greater magnitude compared to higher doses of MA which is suggestive of an inverted U-shaped curve. For example, MA given at 2 mg/kg acutely or 0.25 mg/kg for multiple days seems to have a greater effect on oxidative stress parameters compared to 15 mg/kg acutely or 20 mg/kg for multiple days.

Species	Dose (mg/kg)	No. injections/day	No. of days of drug admin	Route	Ref
Rats	0.25, 0.5, 1 or 2	1	14	ip	(da-Rosa *et al.*, 2012a)
Rats	0.25 or 0.5	1	7	ip	(da-Rosa *et al.*, 2012b)
Mice	1, 2, 10, 20	1	1	sc	(Mori *et al.*, 2007)
Rats	2.5	1	6	ip	(Pang *et al.*, 2013)
Rats	2.5	2 - 10 h apart	1	sc	(Bu *et al.*, 2013)
Mice	3	3 - 3 h apart	1	sc	(Chen *et al.*, 2012)
Mice	4	5 - 2 h apart	1	ip	(Kita *et al.*, 2000)
Rats	5	4 - 2 h apart	1	sc	(Sinchai *et al.*, 2011)
Mice	5 or 10	4 - 2 h apart	1	ip	(Hirata *et al.*, 1995)
Rats	5, 10 or 15	1	1	ip	(Acikgoz *et al.*, 2000)
Rats	7.5	4 - 2 h apart	1	ip	(Fukami *et al.*, 2004)
Mice	7.5	4 - 2 h apart	1	ip	(Shin *et al.*, 2011)
Mice	8	4 - 2 h apart	1	ip	(Shin *et al.*, 2012)
Mice	10	2 - 2 h apart	1	ip	(Thrash-Williams *et al.*, 2013)
Mice	10	1	1	ip	(Toborek *et al.*, 2013)
Mice	10	3 - 2 h apart	1	iv	(Banerjee *et al.*, 2010)
Mice	10	1	1	ip	(Flora *et al.*, 2002)
Mice	10	4 - 2 h apart	1	ip	(Gluck *et al.*, 2001)
Rats	10	2	5	ip	(Koriem *et al.*, 2013)

Species	Dose (mg/kg)	No. injections/day	No. of days of drug admin	Route	Ref
Rats	10	4 - 2 h apart	1	Unknown	(Harold *et al.*, 2000)
Mice	10	5 - 2 h apart	1	ip	(Zhang *et al.*, 2012)
Rats	10	4 - 2 h apart	1	ip	(Yamamoto and Zhu, 1998)
Rats	15	2 - 12 h apart	4	ip	(Zhang *et al.*, 2013)
Rats	20	1/4 - 5 h apart	10	ip	(Moszczynska *et al.*, 1998)

Table 1.13: MA treatment regimes. The range of dosing procedures in MA oxidative stress preclinical studies. n=24 papers.

Route of administration

The route of administration for MA in oxidative stress studies is something that must be considered before beginning the experiment. Among the previous studies, the most common route of administration is ip with 75% of the studies using this route (Hirata *et al.*, 1995, Fukami *et al.*, 2004, Toborek *et al.*, 2013, Zhang *et al.*, 2013) (Figure 1.12) and sc has been used by a small number of studies (17%). It is unclear why some studies have chosen this different route of administration and this is common between both rats and mice. The dosing routine using sc is varied and includes acute and binge dosing but all studies have only looked at one day of dosing. However, although it has been reported that behavioural changes after MA exposure are observed regardless of the route of administration it has been shown that with the ip route, the drug is absorbed more rapidly (Gentry *et al.*, 2004). With sc injections the absorption is slower but the bioavailability of the drug is 100% compared to ip which has a bioavailability of 58% due to hepatic first pass metabolism (Gentry *et al.*, 2004). As these routes of administration show different pharmacokinetic profiles, then the time point of kill after dosing is very important and must ensure that enough time has elapsed in order for its effects to have occurred, and this point will be discussed later in the review (Section 1.5.2). Further investigation into the different routes of administrations used for oxidative stress would be very beneficial. A comparison of these routes would highlight if there are any differences and if the terminal time point needs to be altered based on the route of administration used. Returning again to the clinical situation then we know that oral use or use through inhalation is how MA is most commonly abused in humans (McDonnell-Dowling and Kelly, 2015) but this has been overlooked in preclinical studies to date and so this should also be included in future research.

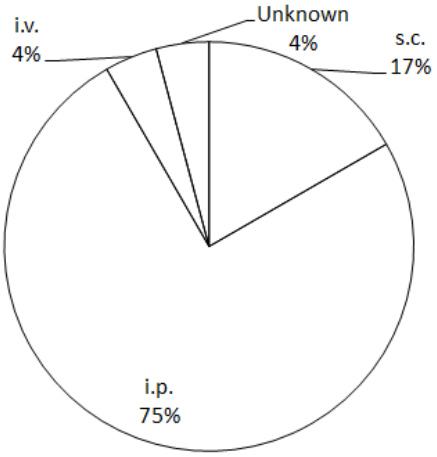

Figure 1.12: MA routes of administration. Pie chart depicting the different routes of administration used for MA in MA oxidative stress preclinical studies.

Animals

The choice of test subject is an important factor to consider when designing an *in vivo* study to investigate MA neurotoxicity and oxidative stress, as these parameters may vary between species. To date, only rodents have been investigated with rats and mice been equally represented. In comparison to mice, rats are less susceptible to stress effects (Collins *et al.*, 1999b) and they provide reliable results that can be translated to humans. However, when we compare the results found for oxidative parameters in rats and mice there are no major differences in these parameters and how they are affected (Table 1.14).

Parameter	Rats	Mice
Monoamines	+	+
Protein carbonyls	+	+
Glutathione system	+	+
Scavenger enzymes (CAT/SOD)	+	+
Lipid peroxidation (MDA/4-HNE)	+	+
NO	+	-
Nitroproteins	+	-
p-tyrosine	+	-
2,3 DHBA	+	-
ROS	-	+
Nrf-2 phosphorylation	-	+
BBB integrity	-	+
TNFα	-	+
AP-1	-	+
COX-2	-	+

Table 1.14: Parameters assessed and altered by MA in rats and mice. The range of oxidative stress parameters assessed and altered after MA administration in rat and mice preclinical studies.

Another factor to consider is the strain of animal. The main rat strains previously used are Wistar and Sprague-Dawley rats however, Fisher 344 rats have also been used. For Sprague-Dawley rats oxidative stress parameters altered include nitric oxide (NO), MDA, CAT, protein carbonyls, GSH, oxidised glutathione (GSSG), GPx, DA and GSH and Wistar rats have shown similar results with altered glutamine, glutamate, gamma-amionbutyric acid (GABA), protein carbonyls, GSH, DA, MDA and SOD. For mice, the most popular strains that have been employed are C57BL/6 mice, Balb/c AnNCrICrIj mice and Swiss-Webster mice and these have all shown similar results for oxidative stress parameters after MA administration.

Therefore it seems that regardless of the species and strain of animal used, alterations in oxidative stress measurements are similar between each animal model.

1.5.2 Measuring oxidative stress

Time point of measurement

The time point after dosing in which the animal is sacrificed is a crucial part of the experimental design and vital to ensure that the optimal time is selected when most oxidative stress parameters can be assessed. There are many time points of sacrifice that have been used in oxidative stress studies after MA (Figure 1.13) and the most common time point of sacrifice is 24 h after dosing.

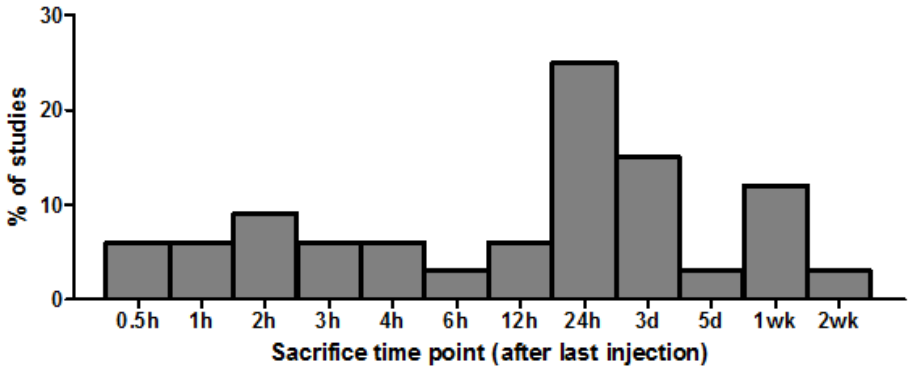

Figure 1.13: Time points of sacrifices. The range of sacrificing time points used in MA oxidative stress preclinical studies.

This time point has been used after various dosing regimens (acute, binge, multiple dosing days) but it is always 24 h after the last MA injection. When the effects are examined at this time point there have been a wide range of alterations to oxidative stress parameters (Table 1.15).

Effect	Parameters				
↑	Nrf-2 phosphorylation	GCS activity	MDA	Protein carbonyl	CAT activity
↓	GSH	GPx activity			

Table 1.15: Effects of MA on oxidative stress parameters after 24 hours. The range of oxidative stress parameters altered 24 h after MA injections in preclinical studies. (Toborek *et al.*, 2013, Koriem *et al.*, 2013, Zhang *et al.*, 2012, Banerjee *et al.*, 2010, Pang *et al.*, 2013).

Therefore all these parameters (GSH, nuclear factor erythroid 2-related factor (Nrf-2), MDA, protein carbonyls, GPx activity and CAT activity) are still measurable 24 h after dosing and again this is regardless of the dosing regimen. Alterations in DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels, tyrosine hydroxylase (TH) activity, TH protein expression, 4-HNE expression, MDA levels, protein carbonyl expression and formation and ROS formation are all still detectable at a sacrifice time point of 3 days after the last MA injection (Chen *et al.*, 2012, Mori *et al.*, 2007, Shin *et al.*, 2011). The studies using this time point looked at acute or binge dosing and the animals only received MA for one day.

Some studies have delayed the point of sacrifice to 1 or 2 weeks after cessation of MA and neurotoxicity was still measurable at these times which suggests long-term damage that is not reversible. After one week Fukami *et al.* (2004) found that MA given at 7.5 mg/kg, 4 times in one day (2 h intervals), decreased levels of DA in rat striatum and these were still apparent. Hirata *et al.* (1995) treated transgenic mice with MA at 5 or 10 mg/kg, 4 times in one day (2 h intervals), and sacrificed the animals 2 weeks later. The 5 mg/kg had no effect but the 10 mg/kg decreased 5-HT uptake sites in the striatum. These studies did not look at any oxidative stress

parameters per se and although neurotoxicity is still evident after 1 or 2 weeks following dosing it suggests that 24 h may be more suitable for examining oxidative stress parameters such as alterations in TBARS, protein carbonyl, GSH and enzyme levels.

Brain regions

Among the preclinical studies that have examined the effects of MA on oxidative stress, many brain regions have been assessed (Table 1.16). Among these brain regions the striatum has been shown to be the most investigated with 79% of these papers examining this region.

Brain region	% of papers
Striatum	79
Hippocampus	38
Cortex	17
Prefrontal cortex	13
Frontal cortex	13
Cerebellum	13
Amygdala	8
Hypothalamus	4
Thalamus	4
NAc	4
Choroid plexus	4
Meninges	4
Brain stem	4

Table 1.16: Brain regions of interest. The range of brain regions used in MA oxidative stress preclinical studies.

As discussed earlier, this brain region is of most interest as it is the primary target of DA-induced effects. When assessing the striatum many oxidative stress parameters have been altered (Table 1.17).

Effect	
↑	↓
TBARS	DA
Protein damage	DOPAC
Lipid damage	GCS activity
GSH	DAT density
Protein carbonyls	HVA
GR activity	5-HT uptake sites
Glrx activity	TH activity
MAC1 immunoreactivity	TH expression
COX-2	VMAT2 expression
DA turnover rate	5-HT
4-HNE expression	3-MT
ROS	5-HIAA
SOD activity	
GPx activity	
TNF-α mRNA levels	
GSSG	
Uric acid content	
2,3 DHBA	
p-tyrosine	

Table 1.17: Effects of MA on oxidative stress parameters in the striatum. The range of oxidative stress parameters altered 24 h after MA injections in preclinical studies. (da-Rosa *et al.*, 2012a, da-Rosa *et al.*, 2012b, Shin *et al.*, 2011, Shin *et al.*, 2012, Gluck *et al.*, 2001, Kita *et al.*, 2000, Acikgoz *et al.*, 2000, Flora *et al.*, 2002, Yamamoto and Zhu, 1998, Mori *et al.*, 2007, Chen *et al.*, 2012, Fukami *et al.*, 2004, Thrash-Williams *et al.*, 2013, Moszczynska *et al.*, 1998, Pang *et al.*, 2013, Harold *et al.*, 2000, Sinchai *et al.*, 2011)

Regardless of the parameter being assessed the striatum is certainly the region that has the most evidence to support the link between oxidative stress and MA use. Other brain regions may be of interest and worth more research to determine their

possible association. For example, the dopaminergic tracts in the brain are not limited to the striatum and project to regions such as the hypothalamus, amygdala and the frontal cortex. The prefrontal cortex has shown promising results with a few studies examining oxidative stress in this region. da-Rosa *et al.* (2012a) showed increased carbonyl formation and TBARS formation in the prefrontal cortex after chronic MA dosing and Bu *et al.* (2013) showed decreased 5-HT and DA levels, and evidence of oxidative stress and membrane disruption after binge dosing of MA at 2.5 mg/kg. Bu *et al.* (2013) also looked at the hippocampus and found similar changes for oxidative stress and membrane disruption. As MA is known to also exert its neurotoxic effects at 5-HT terminals then the projections of the raphe nuclei may also be of interest such as the cortex, hippocampus and hypothalamus.

Parameter/targets to use

There are many ways of measuring oxidative stress (Table 1.18) and to date the methods used have differed between studies but also in the findings reported. There are only a few human studies that have looked at oxidative stress after MA abuse, which have begun to appear recently in the literature. However, among these studies only one study has looked at brain samples and the remaining studies have looked at blood samples. This is important to bear in mind as the use of blood samples may not represent the events occurring in the CNS.

Mitochondrial complex-I	CAT	GST	g-GTP
ROS	Protein carbonyls	GPx	G6PD
SOD	DA, DOPAC, HVA	DAT	GSH
Tight junction proteins	3-MT, 5-HT 5-HIAA	MAC1	ABTS
BBB permeability	COX-2 protein	TH	FRAP
ADMA	Uric acid	4-HNE	GCS
NOS	5-HT uptake sites	AP-1	GGT
Ascorbate	MDA/TBARS Nitroproteins	NF-αB	GR
		TNF-α	Glrx
		Vitamin E	Hydroxyl radicals

Table 1.18: MA oxidative stress targets. The range of parameters and targets used in MA oxidative stress preclinical studies.

Measuring MDA or TBARS levels has shown to be the most common measure in human studies and this has only been measured in blood samples of MA abusers. Solhi *et al.* (2014) took blood samples from people that were regularly using MA (at least once a day for last six months) and found that MDA levels were increased in the blood. However in another study by Walker *et al.* (2014) with people that had used MA for 10.2 ± 7.0 years, they found that there was no difference in TBARS levels; however the authors suggest that this is due to the sample sizes and so this may explain the difference in results. Other parameters that have been used as markers of oxidative stress in humans include oxidative DNA damage using single cell gel electrophoresis in blood samples of MA users and Winhusen *et al.* (2013) found increased oxidative DNA damage in MA users even after a period of abstinence. In human post-mortem samples (although rare) parameters such as levels of GSSG, GPx, GR, glutathione-stransferase (GST), G6PD and CuZnSOD activity have been assessed and MA abusers that were using MA for at least 1 year showed

that there MA was associated with increased CuZnSOD activity and GSSG levels in the caudate nucleus (Mirecki *et al.*, 2004a).

In preclinical studies the same parameters have also been assessed but there are also many others (Table 1.18). For example protein carbonyl levels (Shin *et al.*, 2011), ROS production (Thrash-Williams *et al.*, 2013), nitric oxide synthase (NOS) activity (Zhang *et al.*, 2013), CAT levels (Koriem *et al.*, 2013), SOD activity (Acikgoz *et al.*, 2000) and cyclooxygenase-2 (COX-2) protein expression (Kita *et al.*, 2000). All of these targets have shown significant results which have highlighted a link between MA use and alterations in many oxidative stress parameters. Of all parameters and targets that have been studied preclinically, the most common and perhaps the most promising are those which have focused on the glutathione antioxidant system. This is most likely due to the glutathione antioxidant system being the largest part of the antioxidant defence system and therefore examining this system gives a clear reflection of the oxidative stress levels in the brain. The parameters measured here include total GSH levels, GR activities, GPx activities, G6PD and g-GTP levels (Moszczynska *et al.*, 1998). This system has been assessed in over 40% of MA oxidative stress investigations and generally have shown decreased total GSH levels in the striatum (Moszczynska *et al.*, 1998), increased GPx activity in the striatum and frontal cortex (Flora *et al.*, 2002), decreased GSH levels (Banerjee *et al.*, 2010), decreased GR activity in the thalamus (Pang *et al.*, 2013) and Glrx activity in the thalamus and striatum (Pang *et al.*, 2013).

1.5.3 Antioxidant treatments

Type of antioxidant

The use of antioxidant treatments to combat or attenuate the effects of MA neurotoxicity dates back to the mid-1980s. Wagner *et al.* (1985) used ascorbic acid as an antioxidant pre-treatment before MA dosing. MA was administered for 4 days at 25 mg/kg and caused a long-lasting depletion of DA and 5-HT however, ascorbic acid pre-treatment (100 mg/kg, 30 min before each MA treatment) attenuated this neurotoxic effect. A wider range of antioxidants were again tested by De Vito and Wagner (1989) including ethanol, mannitol and vitamin E and each of these pre-treatments attenuated the depletions of DA and 5-HT in the striatum. Since this time, a number of antioxidants and compounds have been examined to test their effectiveness to attenuate MA neurotoxicity by targeting oxidative stress (Table 1.19).

Antioxidant	Preventative/ Therapeutic	Dose (mg/kg)	Route	Timing	Ref
Caffeic acid	Preventative	100 or 200	ip	1 d before MA	(Koriem *et al.*, 2013)
Rottlerin	Preventative	1.5 or 3.0 μg	Microinfused	Once a day for 5 days and 4-h and 0.5-h before MA	(Shin *et al.*, 2012)
Lithium	Preventative	47.5	ip	Twice a day for 7 d or 14 d	(da-Rosa *et al.*, 2012b)
Valproate	Preventative	200	ip	Twice a day for 7 d or 14 d	(da-Rosa *et al.*, 2012b)
N-acetylcysteine amide	Preventative	250	ip	30 m before MA	(Zhang *et al.*, 2012)
Sulforaphane	Preventative and Therapeutic	10	ip	30 m before MA, 12 h after first SFN and 2 daily SFN for 2 days	(Chen *et al.*, 2012)
Gastrodia elata Bl	Preventative	500 or 1000	po	Twice a day for 6 d until 90 m before MA	(Shin *et al.*, 2011)
N-acetylcysteine amide	Preventative	250	ip	30 m before MA	(Banerjee *et al.*, 2010)
N-acetylcysteine	Preventative	1, 3, 10 or 30	ip	30 m before MA	(Fukami *et al.*, 2004)
Phenylbutylnitrone	Preventative and Therapeutic	150	ip	With first and third MA	(Yamamoto and Zhu, 1998)

Table 1.19: Antioxidants and compounds previously used. The range of dosing procedures for antioxidants and compounds used in MA oxidative stress preclinical studies. n=9 papers.

Figure 1.14 and table 1.20 highlight where in the oxidative stress pathway that these various antioxidants and compounds have had effect and what parameters they have attenuated after MA exposure. The main parameters that have been attenuated include GSH, GPx, MDA, CAT and DA levels. It is important to note that these include compounds which are not known antioxidants and therefore these compounds can also have non-antioxidant mechanisms of action. This section will look at how these treatments have attenuated MA-induced neurotoxicity by focusing solely on the oxidative parameters that have been altered.

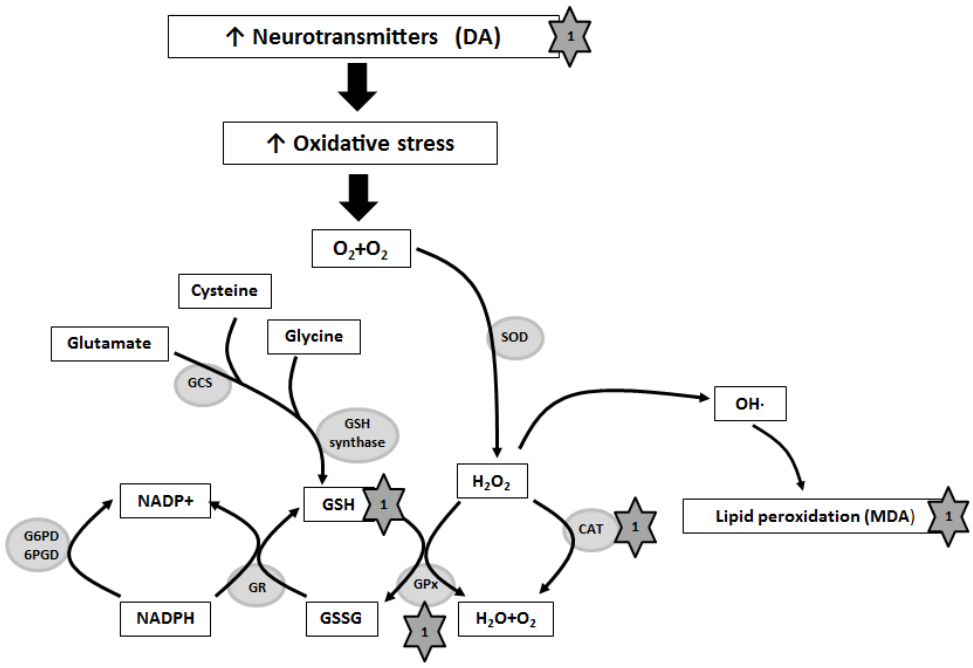

Figure 1.14: Treatment targets in the oxidative stress pathway. The implications of treatments on oxidative stress. 1 indicates the parameters that treatments have attenuated. SOD; Super Oxide Dismutase, MDA; Malondialdehyde, CAT; Catalase, DA; Dopamine, GSH; Glutathione, GCS; Glutamylcysteine synthetase, GSSG; Oxidised glutathione, GR; Glutathione reductase, GPx; Glutathione peroxidase, G6PD; Glucose-6-phosphate dehydrogenase, 6PGD; 6-phosphogluconate

dehydrogenase, NADPH; Nicotinamide Adenine Dinucleotide Phosphate-Oxidase, OH; Hydroxyl Radical, H_2O_2; Hydrogen Peroxide. (Adapted from Halpin *et al.* (2014), Guo and Chen (2012), Isagenix International (2015)).

Antioxidant/Compound	Targets/Parameters altered
Caffeic acid	GSH, GPx, MDA, CAT, PC
Rottlerin	MDA, PC
Lithium	TBARS, PC
Valproate	TBARS, PC
N-acetylcysteine amide	GSH, GPx, MDA, CAT, PC
Sulforaphane	DA, DAT
Gastrodia elata Bl	DA, ROS, MDA, PC
N-acetylcysteine	DA
Phenylbutylnitrone	DA

Table 1.20: Antioxidants and compounds previously used and their targets in the oxidative stress pathway. The implications of treatments on oxidative stress parameters. MDA; Malondialdehyde, CAT; Catalase, DA; Dopamine, GSH; Glutathione, GPx; Glutathione peroxidase, DAT; Dopamine Transporter, ROS; Reactive Oxygen Species, TBARS, Thiobarbituric acid reactive substances, PC; Protein Carbonyls.

N-acetylcysteine is a precursor of glutathione and it acts as an antioxidant which has been shown to attenuate the MA-induced decrease of DA in the striatum in a dose-dependent manner when given as a pre-treatment (Fukami *et al.*, 2004). However this study did not look at any oxidative stress parameters and so it is unclear that at these low doses of the antioxidant has restored these parameters to normal levels. N-acetylcysteine as a treatment has also shown to be problematic in that it can have side effects and the bioavailability is very low (Banerjee *et al.*, 2010). Therefore the

use of N-acetylcysteine amide which is a modified form of N-acetylcysteine, has been reported as a more effective treatment in neurotoxic cases as it can penetrate cell membranes and the blood brain barrier (BBB) (Atlas, 1999). Banerjee *et al.* (2010) and Zhang *et al.* (2012) used N-acetylcysteine amide to see if it has the ability to protect against oxidative stress. N-acetylcysteine amide as a pre-treatment at dose of 250 mg/kg was able to restore GSH, GPx, MDA, CAT and protein carbonyl levels and therefore protect against oxidative stress. Caffeic acid is an α-tocopherol protectant in low density lipoprotein and Koriem *et al.* (2013) evaluated the antioxidant ability of caffeic acid to see if it could attenuate MA-induced oxidative stress and DNA damage. MA was shown to decrease GSH and GPx levels and also increase MDA, CAT and protein carbonyl levels. Pre-treatment with caffeic acid at 100 or 200 mg/kg restores these oxidative stress parameters to normal levels in the hypothalamus. This again highlights the hypothalamus as a possible region of interest for future investigations. This restoration to normal levels was similar for both doses for all parameters and the restored levels were both comparable to the control animals so was therefore not dose-dependent and the same effect can be seen in the low dose of caffeic acid. Valproate is used as an antiepileptic and as a treatment for bipolar disorder but when given at 200 mg/kg da-Rosa *et al.* (2012b) showed that valproate prevented MA-induced protein damage and lipid damage (restored TBARS and protein carbonyl levels) in various brain regions. Phenylbutylnitrone is known to work as an oxygen radical spin trapping agent and when given at 150 mg/kg it restored DA levels in the striatum of MA exposed animals to that of control animals (Yamamoto and Zhu, 1998). However this study did not look at any oxidative stress parameters and so it is unclear if it has restored antioxidant parameters to normal levels. Although many of these compounds have

shown significant results in preventing or attenuating the effects of MA-induced oxidative stress there are very few studies to compare with, each one using a different antioxidant and with a different dosing regimen. In order for these antioxidants to be successful in attenuating or preventing MA-induced neurotoxicity then we must consider how best to deliver these. The next sections will look at how these are administered including dosing regimen, time of exposure and route of administration.

Dosing regime

The frequency of antioxidant dosing or the amount of antioxidant injections given on each day varies between each study. The most common frequencies are single daily doses (Koriem *et al.*, 2013, Zhang *et al.*, 2012, Banerjee *et al.*, 2010, Fukami *et al.*, 2004) or 1-2 doses for multiple days (Shin *et al.*, 2012, da-Rosa *et al.*, 2012b, Shin *et al.*, 2011) (Table 1.19). The number of days in which the animal receives the antioxidant can also vary among studies. One day of administration is generally the most common dosing routine and this is combined with a single injection in one day. When two injections are given in a day this is most commonly given for several days. Therefore the main strategies that have been previously employed are similar to that of the MA dosing routines and are focusing on either chronic/long term dosing or acute dosing.

Dosing timings	% of papers
With MA	9
30 m before MA	46
1-h before MA	9
4-h before MA	9
1 d before MA	18
Twice daily for 3 d before MA	9
Twice daily for 6 d before MA	9
Twice daily for 7 d before MA	18
Twice daily for 14 d before MA	18

Table 1.21: Dosing routines. The range of antioxidant injection routines used in MA oxidative stress preclinical studies.

Chronic dosing (multiple days of dosing) regimes that have been used ranges from 3 days of dosing to 14 days of dosing and table 1.21 shows all the routines previously used. Giving the antioxidant just once has shown to be effective at restoring DA levels, GSH, GPx, MDA and protein carbonyl levels after different MA treatments such as long term MA dosing at 10 mg/kg (Koriem *et al.*, 2013), binge dosing of MA at 10 mg/kg (Zhang *et al.*, 2012, Banerjee *et al.*, 2010) and binge dosing of MA at 7.5 mg/kg (Fukami *et al.*, 2004). Therefore even with an acute dose of the antioxidant this is still effective at protecting the brain against oxidative stress, albeit this is given as a pre-treatment before the MA administration.

Time of exposure

Although the antioxidant treatments have been shown to attenuate oxidative stress when given as a pre-treatment we must consider if it can be given as an additional treatment during MA abuse i.e. if a human is abusing MA can the antioxidant treatment combat oxidative stress if administered alongside the drug. Some studies

have considered this and have tried to examine the therapeutic effects of antioxidants as well as the preventative effects (Table 1.21). Chen *et al.* (2012) used a treatment schedule with sulforaphane to examine the prophylactic and therapeutic effects of this antioxidant in mice. Sulforaphane was given at 10 mg/kg and then 30 min after, MA was administered 3 times at 3 mg/kg (3 h intervals). Then sulforaphane was administered again 12 h after the first sulforaphane. Two daily injections (12 h intervals) of sulforaphane were then given for two consecutive days. Treatment with sulforaphane alone did not have any effect on DA and DOPAC levels however, sulforaphane attenuated the reduction of striatal DA and DOPAC when given before and after the MA treatment. Although this result supports the idea of antioxidant treatment for therapeutic use it cannot be sure that the pre-treatment alone of sulforaphane is having the main effect here as we have seen previously that one acute pre-treatment can prevent oxidative stress. Ideally other groups should be included that have just pre-treatment with the antioxidant and also a group that has just dosing after the MA treatment. Also no oxidative parameters have been assessed. More studies are needed to determine the potential therapeutic effects of antioxidants and a comprehensive study of all antioxidant parameters with this treatment regime needs to be performed. A final time-related aspect to consider is after MA exposure and the potential for antioxidants to restore functioning after MA exposure has occurred. This has not been previously investigated.

Route of administration

The route of administration for the antioxidant in MA oxidative stress studies is something that must be considered before beginning the experiment. Among these studies, the most common route of administration is intraperitoneal injection (ip)

with 82% of the studies using this route (Koriem *et al.*, 2013, da-Rosa *et al.*, 2012a, Fukami *et al.*, 2004, Yamamoto and Zhu, 1998) (Figure 1.15).

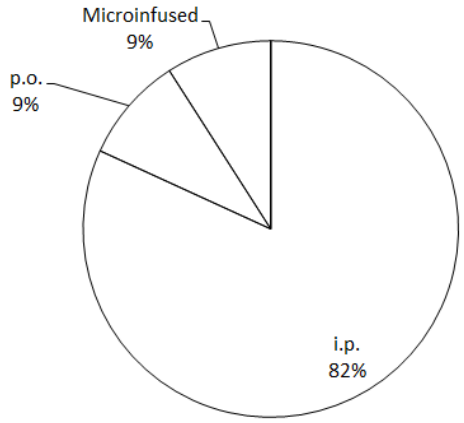

Figure 1.15: Antioxidant routes of administration. Pie chart depicting the different routes of administration used for antioxidant treatments in MA oxidative stress preclinical studies.

This choice of administration route is similar to that of the MA administration and this choice is more than likely due to the ease of administration and rapidness of absorption using ip rather than how best the drug might be administered. Thinking of how best to administer the antioxidant 2 things need to be considered; is the antioxidant a preventative or therapeutic treatment (i.e. is it before or during the MA treatment) and also how will this be administered clinically. To choose a route of administration one needs to consider that in humans, treatments and drugs are most commonly taken by ingesting the drug in tablet or powder form. Therefore, if a treatment becomes available to attenuate the toxic effects of MA then one needs to ensure that it can be given orally and that it will have the same beneficial effects. The use of an oral route of administration is rare (Figure 1.15). To our knowledge, only one preclinical study has used po (oral) to deliver the antioxidant. Shin *et al.*

(2011) gave the methanol extract of gastrodia elata Bl (antioxidant) as a pre-treatment to MA at 500 or 1000 mg/kg twice a day for 5 days until 90 m before MA treatment. They found that gastrodia elata Bl inhibited MA-induced pathologic oxidative changes. Further investigation into the comparison of the different routes of administrations used would be very beneficial and would tell us if there are any differences and also as there are no sc studies, this route should also be included in a comparison study.

1.6 Conclusions

The previous sections have focused on the range of experimental parameters that exist in a MA oxidative stress study. From looking at the various study designs that exist it is clear that there is a large diversity among these studies and so the interpretation and comparison of these results is challenging. Our recommendations for the experimental parameters in a MA oxidative stress study are outlined in figure 1.16 and this acts as a guideline for conducting these studies. For these studies, the dose, route of administration, duration and frequency of exposure are all pivotal to the study design.

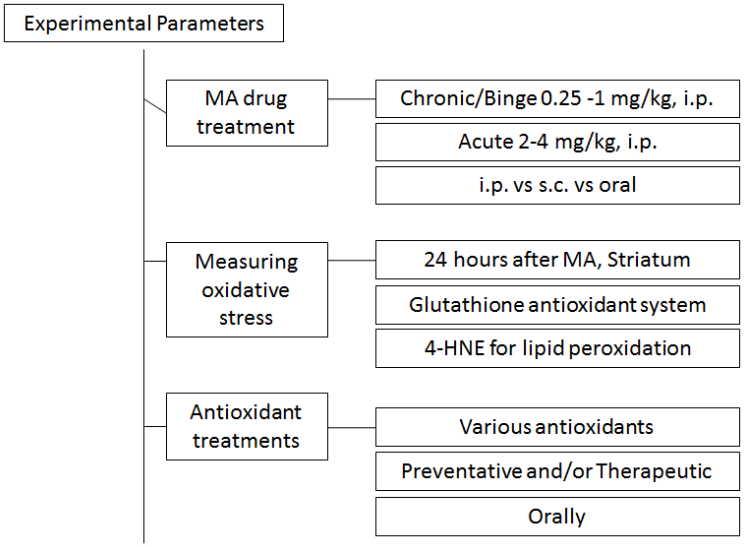

Figure 1.16: Recommended experimental variables. Chart depicting the ideal experimental variables for a MA oxidative stress study.

The recommended preclinical study investigating the effects of MA exposure on oxidative stress in a rodent would involve using a dose of 0.25 to 1 mg/kg for multiple days (binge or chronic dosing) or a dose of 2 to 3 mg/kg for one day (acute dosing). These are doses that have not only yielded the greatest results for oxidative stress so far but they are considered clinically relevant doses and so it allows for comparison to what is happening in the human situation. MA should be administered via oral gavage to again mimic the clinical scenario but a comparison study of different routes of administration (ip, sc and gavage) would be of great benefit to see how these may differ when it comes to measuring the oxidative stress parameters. Multiple exposure durations of MA should be investigated that look at each possible clinical pattern of exposure that may exist for example a long-term chronic user, a recreational user, an infrequent user and also a user that has become abstinent from the drug. With the increasing prevalence of MA use during pregnancy, preclinical

studies should also investigate the consequences of *in utero* MA exposure on oxidative stress in the offspring as this remains unstudied at present.

The recommended measurements of oxidative stress in a preclinical study investigating the effects of MA exposure would involve a sacrifice time of 24 h after the last MA exposure. However, it is worth noting that we previously showed at 1 or 2 weeks after cessation of MA, neurotoxicity was still measurable at these times. As mentioned earlier oxidative stress parameters have not been measured in this field after 24 h and this may be of interest to see are these changes reversible or is long-term damage observed. Therefore, a study investigating sacrifice times may reveal some interesting findings. The main brain region of interest would be the striatum but there is sufficient evidence to show that other brain regions may be of interest such as the hippocampus, hypothalamus, amygdala and frontal cortex. Although TBARS/MDA levels have shown the most consistent results to date both clinically and preclinically, these assays are known to be problematic in that they are unreliable, non-specific and are quite labour intensive. Positive results can be produced in this assay for lipid peroxidation with practically any antioxidant (or superfluous relic) (Jacob *et al.*, 2008). For this reason, in general oxidative stress assays (regardless of the insult given) 4-HNE is more commonly used as it provides more reliable results yet this has been overlooked previously. Other parameters that have not been previously investigated include 8-Hydroxydeoxy guanosine which is a biomarker of ROS-induced DNA damage and 8-nitroguanine levels that are used to evaluate oxidative DNA damage and these are quite common in other oxidative stress studies. These targets and parameters as well as the glutathione antioxidant system warrant more investigation in future studies.

The use of antioxidant treatments is still in its early days and therefore optimisation of administration of these could be beneficial. Regardless of the antioxidant selected there are many results. For these studies, the dose, route of administration, duration and frequency of exposure are again all pivotal to the study design (Figure 1.16).

The recommended preclinical study investigating the effects of MA exposure on oxidative stress in a rodent would involve administering a suitable antioxidant dose via oral gavage. The oral route of administration of the antioxidant is a very important factor as this allows a more realistic extrapolation to the human situation. Multiple exposure durations of the antioxidant should be investigated to see the potential of the compound as a preventative treatment (pre-treatment before MA) or as a therapeutic treatment (alongside MA or after MA) and which may be better suited.

The use of these recommendations mean that results will be more comparable between papers and that future results generated will give us a true understanding of what might be happening in the clinical scenario.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

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Chapter 2:

Methamphetamine exposure during pregnancy at pharmacological doses produces neurodevelopmental and behavioural effects in rat offspring

McDonnell-Dowling, K., Donlon, M. & Kelly, J. P. 2014. Methamphetamine exposure during pregnancy at pharmacological doses produces neurodevelopmental and behavioural effects in rat offspring. *International Journal of Developmental Neuroscience,* 35, 42-51.

Abstract

In recent years MA use has become more prevalent, and of particular concern is the growing popularity among women of childbearing age. However, to date, studies examining MA effects on the developing offspring in laboratory animals are limited. Thus, the aim of this study was to determine if *in utero* MA exposure in rats at pharmacological doses can have a negative impact on neonatal neurodevelopment and behaviour. Pregnant Sprague-Dawley dams (n=10 dams/group) received MA (0, 0.625, 1.25, 2.5 mg/kg) once daily via oral gavage from gestation day 7-21. Maternal body weight, food and water consumption were recorded daily. A range of standard neurodevelopment parameters were examined in the offspring during the neonatal period. There were no neurodevelopmental deficits observed with offspring exposed to 0.625 mg/kg MA, in fact, there were enhancements of neurodevelopment in some parameters at this low dose. However, exposure to the 1.25 mg/kg MA dose resulted in significant impairments in surface righting reflex and forelimb grip in both sexes. Exposure to the 2.5 mg/kg MA dose resulted in a significant reduction in ano-genital distance in males, and in both sexes resulted in delayed fur appearance and eye opening, impairments in surface righting reflex and negative geotaxis, and a reduction in body length. In conclusion, this study demonstrates that pharmacologically relevant doses of MA can have profound dose-related effects on neonatal outcome. If extrapolated to the clinical scenario this will give cause for concern regarding the risks associated with this drug of abuse at relatively low doses.

2.1 Introduction

The use of illicit drugs has shown a steady increase in prevalence in young people and this is particularly evident in the last five years in the US (Substance Abuse and Mental Health Services Administration, 2013). Between 2008 and 2012, reports showed that in the US, there was an increase in illicit drug use in persons aged between 18 and 25 from 19.7% to 21.3% (Substance Abuse and Mental Health Services Administration, 2013). Substance abuse in women typically occurs during the childbearing age (Anderson and Choonara, 2007) and thus the prevalence of substance abuse during pregnancy is increasing, which in turn means that the amount of drug-exposed babies is increasing. There are many potential health risks and adverse effects associated with substance abuse during pregnancy for not only the foetus but for the mother as well (Schempf and Strobino, 2008, Good *et al.*, 2010).

The amphetamine-like stimulants (ALS) include AMP, MA and MDMA and these are appealing to drug users due to their low cost, ease of use and availability. Early human studies found that prenatal MA-exposed infants exhibited poor alertness, poor feeding and lethargy (Dixon, 1989). Adverse effects previously reported include low birth weights (Smith *et al.*, 2006) and a higher incidence of cleft lip, ambiguous genitalia and anencephaly (Good *et al.*, 2010). Similar studies also showed altered neurocognitive performance in that MA-exposed children had lower verbal memory, long-term spatial memory, sustained attention and visual motor integration (Chang *et al.*, 2004). Although each of these studies looks at prenatal MA exposure, the patterns of use of MA and doses of MA used can differ greatly between pregnant females. In 2010, a study from the U.S. in areas with high reports of MA use showed that in the first trimester, 14.7% of females in this study took MA 1-2 times during

pregnancy whereas 23.6% of the females took MA almost every day. Again this pattern of use changed as females progressed through the pregnancy and moved into the second and third trimester with 29.3% of females staying on a consistently high frequency of use and 35.6% of females actually decreasing their frequency of use of MA during this period (Della Grotta *et al.*, 2010).

The use of animal models has been essential in helping to understand the neurodevelopmental and behavioural consequences of drug exposure during pregnancy and breastfeeding (Thompson *et al.*, 2009b). An advantage of using an animal model is being able to explore any consequences of this drug exposure while eliminating the aforementioned limitations that are common in clinical studies. Many early animal studies aimed to elucidate the short and long-term effects of prenatal MA exposure. Adverse effects that have been reported with prenatal MA include cleft palate (Yamamoto *et al.*, 1992), retinal eye defects (Acuff-Smith *et al.*, 1992) and delayed physical growth and motor development (Cho *et al.*, 1991). Behavioural consequences have been seen in early adulthood of rat offspring prenatally exposed to MA including a higher pain score in the formalin test suggesting a long lasting hypersensitivity to pain (Chen *et al.*, 2010).

However to date, the current literature with regards to prenatal MA exposure has failed to reflect an accurate clinical experience in aspects such as route of administration, dose of MA and time and duration of MA exposure. For example, the most common route of administration in these animal studies is sc injection with 88% of the existing studies using this route (Slamberova *et al.*, 2011b, Grace *et al.*, 2010, Vorhees *et al.*, 2009). Ip injection has also been used in some studies (Siegel *et*

al., 2010, Wong *et al.*, 2008, Inoue *et al.*, 2004) and this shows the same advantages and disadvantages as the sc route although the drug is absorbed much quicker. In contrast in humans, MA is most commonly taken orally where the drug is ingested in powder or tablet form, or by inhalation where the drug is smoked (U.S. Department of Health and Human Services, 2006). In preclinical studies, in order to accomplish an oral route of administration, MA has previously been administered via the drinking water (Tonge, 1973). However, as animals are sensitive to potent smells, they may reject the drug in this form.

Among the different dosing regimens, throughout the entire gestation is the most commonly used duration of exposure (Yamamotova *et al.*, 2011, Bubenikova-Valesova *et al.*, 2009, Schutova *et al.*, 2009a). This dosing regimen relates to the clinical situation where the mother is abusing MA everyday throughout pregnancy. This is consistent with a study by Della Grotta *et al.* (2010) that showed 55% of pregnant MA users did not change their pattern of use over their pregnancy. It is unknown if the women that decreased their use during pregnancy were able to maintain this or relapsed and returned to their original patterns of use. For this reason, many studies have looked at the postnatal period as a potential time of exposure for the offspring. Unfortunately, some previous studies have administered MA directly to the offspring during the postnatal period (Gomes-da-Silva *et al.*, 2004, Williams *et al.*, 2004, Schaefer *et al.*, 2008). These studies are trying to target a certain point of development in the offspring, which correlates to the third trimester of pregnancy in humans. However, in the clinical situation we know that the offspring are exposed indirectly to the drug through breast milk and not by direct exposure via injection and so, the same should be performed in preclinical studies.

MA abusers generally use a dose starting at 20 mg with a common MA dose being 30 mg (Golub *et al.*, 2005). When extrapolating this dose back to a preclinical model, the use of an allometric scale (Reagan-Shaw *et al.*, 2008) takes into account the body weight and the body surface area of the human and the animal. Therefore, the MA dose to use in a preclinical model (rat) would be 2.5 mg/kg. In order to accurately model the clinical scenario, then doses within this range would be most appropriate and this should be administered directly to the mother via oral gavage during the gestation period.

The aim of this study was to use a clinically relevant animal model of MA abuse during pregnancy to elucidate the consequences of exposure at a variety of doses. The hypothesis is that prenatal MA exposure at pharmacological doses can have a negative influence on the neurodevelopment of the rat offspring.

2.2 Materials and Methods

2.2.1 Animal Housing

Adult male (275–300 g) and female (250–275 g, approx. 3.5 months old) Sprague-Dawley rats were used for this study. All females were bred in house and housed in groups of three from the beginning of the study. All males came from Charles River (Kent, U.K.) and from the day of arrival, males were housed singly. All animals were housed in plastic bottom cages with appropriate bedding material and were handled daily. All female rats were housed singly after mating occurred and additional nesting materials were also given. All animals were maintained under standard laboratory conditions under artificial 12-h light/dark cycle (lights on from 08:00h) and temperature was maintained at 21-24^{o}C with relative humidity at 35-

60%. Animals had free access to food and water throughout (food and water consumption were measured once daily at 14.00h). Following littering, the rat pups were all housed with their biological mothers until PND 21 when the pups were weaned. The pups stayed with their siblings for 7 days until PND 28, at which point they were then separated by sex; littermates of the same sex remained together throughout adulthood. All experiments were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway (12/NOV/07) and in compliance with the European Communities Council directive 86/609.

2.2.2 Mating

For this research project, 72 female rats and 24 male rats were acquired to ensure adequate numbers of females became pregnant and that there was a male:female ratio of 1:3 for mating. The animals were left undisturbed and allowed to habituate for one week before the study began. Each female was allowed to habituate to the 2 other female cagemates and then each cage of female rats (3) were housed overnight with one sexually mature male rat ensuring that the same females remained together. At the beginning of the light phase the following morning, vaginal smears were taken from all females to check for the presence of sperm. All smears were examined under a light microscope. GD 1 was deemed to be the day that sperm was present in the smear, at which point the female was then removed and singly housed.

2.2.3 Gestation Period and Deliveries

The females were checked daily prior to the start of the dosing period (GD 7-birth) and the females were weighed daily. The expected day of delivery (birth) is GD 21-22 (Daston *et al.*, 2004). Deliveries that occurred before 17.00h were considered to

have their and PND 1 on this day and deliveries that occurred after 17.00h were considered to have their PND 1 on the following day. Offspring in each litter were checked and counted for the week after delivery to monitor for pup mortalities. The pups were randomly culled to 10 per litter on PND 1 with a litter ratio of 50:50, males to females whenever possible. Two males and two females were selected for neonatal testing from each litter in order to avoid litter effects. These four pups represented the average weight of the litter. Each of these pups was injected intradermally with black India ink in the footpad for unique identification purposes in a litter.

2.2.4 Drug Treatment

The first dosing day corresponded with GD 7; this timeline corresponds to the second trimester of human pregnancy and to the human prenatal development of the CNS (Daston *et al.*, 2004). Methamphetamine HCL was purchased from Sigma Aldrich (Wicklow, Ireland; M8750). Rats were assigned randomly to control or MA treated groups by way of latin square based on body weights (weight gained before mating and since GD 1) and likelihood of pregnancy to ensure even distribution across all groups. The doses of MA given were 0.625, 1.25, 2.5 mg/kg at a volume of 1 ml/kg and controls received the vehicle alone i.e. 1 ml/kg distilled H_2O (dH_2O). These doses were chosen as they relate to clinically relevant doses in the human scenario and the use of an allometric scale takes into account the body weight and body surface area of human versus rat (Reagan-Shaw *et al.*, 2008) therefore maintaining the viability of the project and its comparison to clinical situations. For MA or control treatments, the animals were dosed via oral gavage once daily at 14.00h until dams gave birth. Oral gavage was used as this represents the most

common route of MA administration in humans, but has before been overlooked in preclinical investigations.

2.2.5 Maternal Daily Measurements

Maternal body weight was recorded daily from GD 1 to birth prior to dosing of each rat (between 14.00 and 16.00h). Maternal food and water consumption were also recorded daily from GD 1 to birth for each rat, at similar times each day (between 14.00 and 16.00h).

2.2.6 Development of offspring

The development of the offspring was measured by looking at somatic development and behavioural testing. Somatic development measures the physical maturation of the pup (Mesquita *et al.*, 2007) and behavioural testing looks at tests of sensorimotor development which reflect the maturation of vestibular function (Khan *et al.*, 2004). The day on which each test is performed relates to the time at which this development milestone normally occurs in rats and each test has a specific PND (see below).

Somatic Development of Offspring

Somatic parameters were observed and recorded for selected pups from control and MA groups over a three-week period. These parameters included pinna (ear) unfolding, fur apperance, eye opening, ano-genital distance, body length and body weight. Pinna unfolding was recorded from PND 3, eye opening was recorded from PND 14 and fur appearance was recorded from PND 3 for males and females. The time of first appearance was considered to be the first day of occurrence and for

pinna unfolding and eye opening both pinna or eyes must unfold or open, respectively. Recording of these parameters continued until all pinna had unfolded, eyes had opened and fur was present in all rat pups.

Ano-Genital Distance

Ano-genital distance was measured (for comparison) for possible masculinising or feminising effects of MA. All selected pups were measured to assess the ano-genital distance using a digital calipers.The distance was measured between the base of the genitals and the top of the anus and it was recorded in mm. This was performed on PND 3 and 24 and all selected rat pups within all the litters were tested once on each of these PNDs.

Body Length

Body length was measured for each selected rat pup for control and MA groups on PND 7 and 14 to compare growth sizes. The pups were placed on a flat black surface beside a digital calipers. The length of the pups was regarded as the distance between the tip of the nose and the base of the tail and this was recorded in mm. This was performed on PND 7 and 14 and all selected rat pups within all the litters were tested once on each of these PNDs.

Body Weight

Body weight was measured for each selected rat pup for control and MA groups throughout the neonatal, adolescent and adult periods on PND 1, 2, 4, 8, 11, 15, 18, 21, 29, 36, 43, 50, 57, 64 78, 92, 106 and 113 prior to behavioural testing each day.

Behavioural Testing of Offspring

A variety of behavioural tests were performed on the selected rat pups. Specific PNDs were selected for the behavioural tests and these began at 09.00h on the

appropriate PND. Both mother and pups remained in the home cage room while testing occurred. At the time of testing, the mothers were removed from the home cage and placed in a separate cage. The pups were separated into males and females for testing and were taken directly from the home cage. Unless stated otherwise, the pup was placed back into the home cage after the test and the mother was also returned directly after testing was completed.

Righting Reflex on a Surface

Dynamic tests of sensorimotor development, such as righting reflexes, give an indication of the development of body righting mechanisms (Mesquita *et al.*, 2007). Righting reflex on a surface was tested by placing the rat pups in the supine position on a flat surface and the time taken to turn over and restore its normal prone position (on all fours) was recorded. The time taken to turn over was recorded with a stopwatch (in sec) and the maximum time allowed was 30 sec. A time of 30 sec was given if the pup did not right itself within this period and the test was terminated. The righting reflex test was performed in the same room as the home cage. This test was performed on PND 2, 3, 4 and 5 and all selected rat pups within all the litters were tested once on each of these four PNDs.

Righting Reflex Mid-Air

Righting reflex tests reflect the maturation of vestibular function (Khan *et al.*, 2004). Righting reflex in mid-air was tested by placing the rat pups in the supine position 40 cm above a soft padded surface and releasing them. The position they landed on when reaching the soft pad was observed and recorded. A score was given depending on the position they landed. A score of "1" was given when a pup turned over and restored its normal prone position (on all fours) and a score of "0" was given when the pup did not. The righting reflex test was performed in the same

room as the home cage. This test was performed on PND 12 and 17 and all selected rat pups within all the litters were tested once on each of these PNDs.

Negative Geotaxis

The negative geotaxis test also looks at sensorimotor development (Mesquita *et al.*, 2007). The apparatus consists of a flat timber surface that is inclined at a 30˚ angle and held in place. At the top of this apparatus, there is a rough surface for the rat pups to grip to. The rat pups were placed facing downward at the top of the negative geotaxis apparatus. The time taken to turn 180˚ and face upward was recorded. The time taken to turn around was recorded with a stopwatch (in sec) and the maximum time allowed was 60 sec. A time of 60 sec was given if the pup did not turn within this period and the test was terminated. The negative geotaxis test was performed in the same room as the home cage. This test was performed on PND 9 and 11 and all selected rat pups within all the litters were tested once on each of these PNDs.

Forelimb Grip

In order to test the gripping reflex of the neonates the forelimb grip test was used and this gives a good indication of the strength of the rat pups (Sousa *et al.*, 2006) as well as vestibular function and sensormotor coordination. The forelimb grip apparatus consists of a thin steel bar, which is supported by two adjustable poles. The bar is approx. 20 cm in length, 0.2 cm in diameter and lies 25 cm above the base of the platform where a soft towel was placed to cushion the fall. The rat pups were gripped at the base of the tail and lowered onto the bar to allow it to grasp the bar with its forepaws. When a grip with both forepaws was established, the tail was lowered and released. The length of time the rat was able to hold on to the bar before falling was recorded with a stopwatch (in sec) and the maximum time allowed was 30 sec. A time of 30 sec was given if the pup did not fall within this period and the test was

terminated. The forelimb grip test was performed in the same room as the home cage. This test was performed on PND 14 and 17 and all selected rat pups within all the litters were tested once on each of these PNDs.

2.2.7 Adolescent and Adult Behavioural Testing

A variety of behavioural tests were performed on the selected rats. Specific PNDs were selected for the behavioural tests and these began at 09.00h on the appropriate PND. At the time of testing, the rats were removed from the home cage and placed into the arena. All testing occurred in novel cage rooms outside the home cage room. Unless stated otherwise, the rat was placed back into the home cage after testing was completed. All experimenters were blind to the treatment condition of the animal during testing.

Elevated Plus Maze

The Elevated Plus Maze (EPM) is one of the most widely used tests to assess a wide range of anxiety-related behaviours and has been validated for use in both rats and mice (Sidor *et al.*, 2010). The EPM is based on Mongomery's assumption that a rat has a natural aversion to open areas and heights which conflicts with its investigative tendencies to explore the open arms (Montgomery, 1954). The EPM consists of a plus shape with two open arms (50 cm×13 cm), two enclosed arms (50 cm×13 cm) and a central platform (10 cm×13 cm) made of black polycarbonate plastic. Each arm is supported by a sturdy plastic leg and is raised 46cm above the floor. The two enclosed arms have high walls (30 cm in height) on the sides and ends. To avoid shadows over the arms two 100W bulbs are placed above the open arms with the intensity of light set at 80 Lux in the open arms and 35 Lux in the closed arms. Behaviours were recorded by a camera above the EPM linked to a DVD recorder in

the same room. Ethovision video tracking software was used to track and score behavioural measures; duration of time spent in each zone (centre, open and closed arms), frequency of entries into each zone (centre, open and closed arms), total distance moved and velocity of movements within the EPM. The EPM test was carried out when the weaning stage was finished on PND 29. At the time of testing, the selected rat pups were taken directly from their home cages, weighed and placed in the centre of the EPM facing one open arm. The test lasted for 5 min after which the animal entered straight into the open field test. After each test, the EPM was cleaned before the next animal was placed in the maze.

Open Field

The open field (OF) test (in a similar way to the EPM) is used to assess anxiety-related behaviours, but also examines general locomotor activity. The nature of rodents is to avoid open spaces and bright lights and to remain in the periphery of the OF apparatus (thigmotaxis) (Sousa *et al.*, 2006). The OF apparatus consists of a white, circular base (75 cm diameter) surrounded by wall (43 cm in height). The base of the OF is divided into 10 cm squares by black lines. To avoid shadows in the OF four 100W bulbs are placed above the OF with the intensity of light set at 190-210 Lux. Behaviours were recorded by a camera above the OF linked to a DVD recorder in the same room. Ethovision video tracking software was used to track and score behavioural measures; duration of time spent in each zone (inner and outer), frequency of entries into each zone (inner and outer), total distance moved and velocity of movements within the OF (Simpson *et al.*, 2012). The OF test was carried out on PND 29 and the selected rat pups were taken directly from the EPM and placed in the centre of the OF. The test lasted for 5 min after which the animal was sacrificed. After each test the OF was cleaned before the next animal.

Morris Water Maze

The Morris water maze (MWM) is a test of spatial learning for rats and mice (Vorhees and Williams, 2006). The MWM is assessed by looking at the time an animal takes to find a hidden platform on repeated exposures. The MWM apparatus consists of a tank measuring 2 m in diameter and filled to a depth of 0.3 m with water. The water temperature was between 23-25^{o}C. An escape platform was located in one quadrant of the tank (southwest, SW) and is submerged (2 cm) below the surface of the water (i.e. water filled to 0.32 m) for the acquisition trials. Large geometric shapes (printed in black and white on A4 paper) are placed around the maze to provide visual cues. These cues are held in constant spatial relations throughout the experiments. The apparatus is illuminated by four 100W bulbs placed above the MWM with the intensity of light set at 25 Lux. On the acquisition day 1 (PND 85) the selected rat pups were taken directly from their home cages, weighed and placed into the MWM from varied starting points from Nature Protocols (Vorhees and Williams, 2006), so that it is facing the wall of the pool. The rat is allowed to swim around until it finds the platform up to a max duration of 120 sec. Blocks of four trials are presented to each rat, one block per day (this is the acquisition phase and lasts 4 days). On reaching the platform, each rat is allowed to remain there for 10 sec before being removed from the maze, dried gently using a cotton towel and placed in a recovery cage. If a rat fails to locate the platform within 120 sec, it is guided there by the experimenter and left here for 10 sec. Once the rat is placed in its recovery cage, a timer is set. When the same rat is then removed for its next trial, the inter-trial interval on the timer is recorded. In the acquisition phase, the platform is hidden in the southwest pool quadrant; in the probe trial the platform is absent and the rats are released from a novel release point (northeast, NE).

Behaviours were recorded by a camera above the MWM linked to a DVD recorder in another room. Ethovision video tracking software was used to track and score behavioural measures; distance and velocity travelled within the MWM and time spent in SW quadrant on probe day (testing day). Performance can also be rated on latencies to find the platform.

2.2.8 Statistical Analysis

All figures representing the data from the testing period were constructed using Graphpad Prism 5.0. The data were then analysed using the statistical package SPSS 20.0. Data were firstly assessed to determine if it displayed normality of distribution and homogeneity of variance (Shapiro-Wilks and Levene's test $p>0.05$). This determines whether the data are parametric or non-parametric. The data were also assessed to determine if it displayed sphericity (Mauchly's test $p>0.05$) however if this failed then the Greenhouse-Geisser correction was used (i.e. degrees of freedom corrected). For the parametric data tests used included; repeated measures ANOVA to compare the overall effect for related data, Two-Way ANOVA to compare the effect of treatment groups and sex, One-Way ANOVA and Student-Newman Keuls *post-hoc* tests were used to define where the significance lay. Parametric data includes maternal body weights and food consumption, birth weights, EPM and OF. For the non-parametric data tests used included: Friedman's ANOVA by Ranks to compare the overall effect for related data, Wilcoxon Match-Pairs test to compare the effect of time, Kruskal-Wallis test to compare the effect of treatment groups and Mann-Whitney U tests were used to define where the significance lay. Non-parametric data includes ano-genital distance, body length, righting reflex mid-air and neonatal bodyweight gain. For pinna unfolding, eye opening and fur appearance

the data were taken as counts (present or absent) and for surface righting, negative geotaxis and forelimb grip the data were taken as counts (ability to perform the test or not) and so the Chi-Squared test was performed for these parameters. The level of significance was set at $p<0.05$.

2.3 Results

2.3.1 Maternal Daily Measurements

Maternal Weight Gain

A significant effect of gestation day (time) was found ($F_{(2.59, 85.48)}=561.88$, $p<0.001$) (Figure 2.1) with all groups gaining weight as gestation progressed. No significant effect of treatment was found ($F_{(3, 33)}=0.17$, $p>0.05$) (Figure 2.1). No significant interaction effect of gestation day and treatment was found ($F_{(7.77, 85.48)}=0.49$, $p>0.05$). No significant effect of treatment was found for pre-dosing body weight gain ($F_{(3, 36)}=0.69$, $p>0.05$) or body weight gain in the first week of dosing ($F_{(3, 36)}=1.66$, $p>0.05$), but a significant effect of treatment was found for body weight gain in the second week of dosing ($F_{(3, 36)}=3.24$, $p<0.05$) (Figure 2.1). A *post-hoc* test showed that there were no differences between the treatment groups compared to the control group.

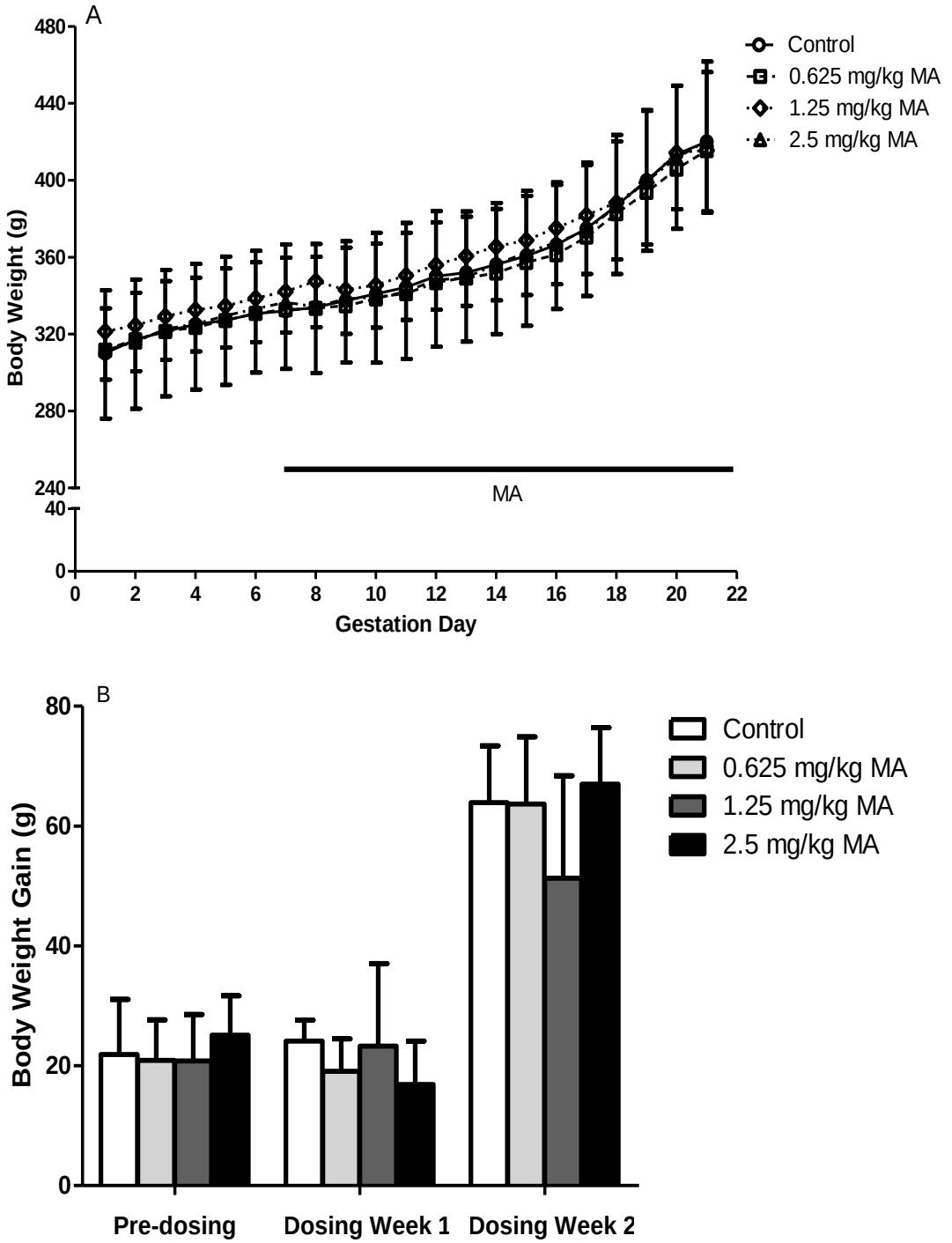

Figure 2.1: Maternal weight gain during gestation. (A) Weight Gain for control, 0.625 mg/kg MA, 1.25 mg/kg MA, 2.5 mg/kg MA groups for each day of gestation (n=10/group). (B) Total Weight Gain from the pre-dosing and post-dosing periods. Line indicates dosing period. Data are expressed as Mean±SD; MA, methamphetamine. See text for details of statistical analyses.

Maternal Food and Water Consumption

A significant effect of gestation day (time) was found for food consumption ($F_{(2.49, 77.08)}$=5.66, p<0.01) and water consumption (Fr=410.95, p<0.001) (data not shown). No significant effect of treatment was found for daily food consumption ($F_{(3, 31)}$=1.53, p>0.05) or daily water consumption (data not shown). No significant interaction effect of gestation day and treatment was found for food consumption ($F_{(7.46, 77.08)}$=0.84, p>0.05) (data not shown). No significant effect of treatment was found for total water consumption during the pre-dosing period (K=0.19, p>0.05), in the first week of dosing (K=2.79, p>0.05) or in the second week of dosing (K=1.97, p>0.05) (data not shown). No significant effect of treatment was found total food consumption during the pre-dosing period ($F_{(3, 36)}$=1.21, p>0.05), in the first week of dosing ($F_{(3, 36)}$=0.72, p>0.05) or in the second week of dosing ($F_{(3, 36)}$=0.59, p>0.05) (Figure 2.2).

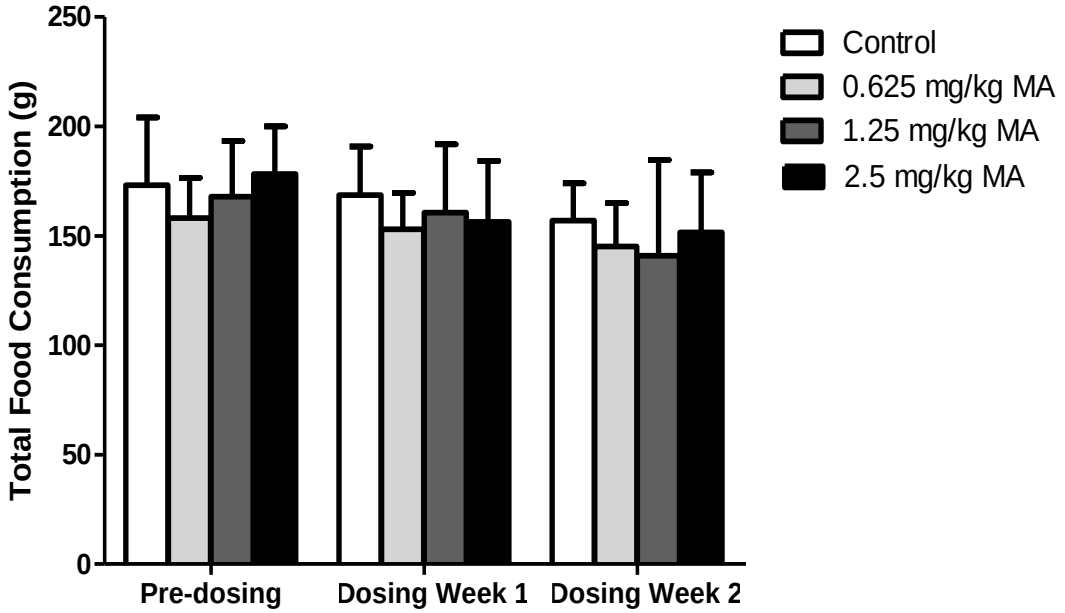

Figure 2.2: Maternal food consumption during gestation. Total food consumption from the pre-dosing and post-dosing periods for control, 0.625 mg/kg MA, 1.25 mg/kg MA, 2.5 mg/kg MA groups (n=10/group). Data are expressed as Mean+SD; MA, methamphetamine. See text for details of statistical analyses.

2.3.2 Somatic Development of Offspring

Birth Weights and Mortalities

A significant effect of treatment was found ($F_{(3, 139)}$=6.13, p<0.01) (data not shown) and a significant effect of sex was found ($F_{(1, 139)}$=7.73, p<0.01) with females (5.8 ± 0.5) being lighter at birth compared to males (6.2 ± 0.5). There was no significant interaction effect of treatment and sex ($F_{(3, 139)}$=0.24, p>0.05). A *post-hoc* test showed that there were no differences between the treatment groups compared to the control group for males or females (data not shown). There was no significant difference between the treatment groups for the number of stillborn pups or pups that were found dead/eaten in the post-natal period (data not shown).

Pinna Unfolding

No significant effect of sex was found on PND 3 (X_1^2=0.52, p>0.05), PND 4 (X_1^2=0.04, p>0.05) or PND 5 (X_1^2=1.01, p>0.01). A significant effect of treatment was found on PND 3 (X_1^2=15.02, p<0.01) and PND 4 (X_1^2=33.68, p<0.001). No significant effect of treatment was found on PND 5 (X_1^2=2.87, p>0.05) (Table 2.1). A *post-hoc* test showed that on PND 3 for males and females the 0.625 mg/kg MA group (p<0.05) had more pups with pinna unfolded than their control groups. On PND 4, 0.625 mg/kg males and females (p<0.01) had more pups with pinna unfolded compared to their control groups.

Drug	PND 3	PND 4	PND 5
Male			
Control	15%	70%	100%
0.625 mg/kg	45%*	100%**	100%
1.25 mg/kg	29%	94%	100%
2.5 mg/kg	10%	50%	95%
Female			
Control	20%	70%	100%
0.625 mg/kg	50%*	100%**	100%
1.25 mg/kg	35%	94%	100%
2.5 mg/kg	15%	55%	100%

Table 2.1: Rat pup pinna unfolding. Percentage of pups with both pinna unfolded for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=17-20/group). Data are expressed as percentage of pups with both pinna unfolded; **p<0.01 vs. relevant control; *p<0.05 vs. relevant control; MA, methamphetamine. See text for details of statistical analyses.

Fur Appearance

No significant effect of sex was found on PND 3 (X_1^2=0.25, p>0.05), PND 4 (X_1^2=3.81, p>0.05), PND 5 (X_1^2=0.34, p>0.05) or PND 6 (X_1^2=0.00, p>0.05). No significant effect of treatment was found on PND 5 (X_1^2=5.25, p>0.05) or PND 6 (X_1^2=8.09, p>0.05). A significant effect of treatment was found on PND 3 (X_1^2=21.23, p<0.01) and PND 4 (X_1^2=18.21, p<0.01) (Table 2.2). A *post-hoc* test showed that on PND 3 for males the 2.5 mg/kg MA group (p<0.01) had less pups with fur than the male control group. On PND 4 for females the 2.5 mg/kg MA group (p<0.05) had less pups with fur than the female control group.

Drug	PND 3	PND 4	PND 5	PND 6
Male				
Control	90%	100%	100%	100%
0.625 mg/kg	90%	100%	100%	100%
1.25 mg/kg	71%	94%	94%	94%
2.5 mg/kg	45%**	100%	100%	100%
Female				
Control	80%	100%	100%	100%
0.625 mg/kg	90%	100%	100%	100%
1.25 mg/kg	81%	94%	94%	94%
2.5 mg/kg	65%	80%*	95%	100%

Table 2.2: Rat pup fur appearance. Percentage of pups with fur for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=18-20/group). Data are expressed as percentage of pups with fur; **p<0.01 vs. relevant control; *p<0.05 vs. relevant control; MA, methamphetamine. See text for details of statistical analyses.

Eye Opening

A significant effect of sex was found on PND 14 (X_1^2=4.80, p<0.05) with males pups having less eyes open compared to female pups. No significant effect of sex was found on PND 15 (X_1^2=1.25, p>0.05), PND 16 (X_1^2=0.00, p>0.05) or PND 17 (X_1^2=0.00, p>0.05). A significant effect of treatment was found on PND 14 (X_1^2=13.30, p<0.05), PND 15 (X_1^2=21.19, p<0.01), PND 16 (X_1^2=24.38, p<0.001) and PND 17 (X_1^2=15.69, p<0.01) (Table 2.3). A *post-hoc* test showed that on PND 14 for females the 1.25 mg/kg MA group (p<0.05) had more pups with eyes open than the female control group. On PND 15 and PND 16 for females and males respectively, the 2.5 mg/kg MA group (p<0.01) had less pups with eyes open than their control groups.

Drug	PND 14	PND 15	PND 16	PND 17
Male				
Control	20%	85%	100%	100%
0.625 mg/kg	20%	100%	100%	100%
1.25 mg/kg	29%	77%	100%	100%
2.5 mg/kg	6%	61%	72%**	89%
Female				
Control	15%	100%	100%	100%
0.625 mg/kg	40%	100%	100%	100%
1.25 mg/kg	47%*	88%	88%	100%
2.5 mg/kg	22%	72%**	83%	89%

Table 2.3: Rat pup eye opening. Percentage of pups with both eyes open for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=17-20/group). Data are expressed as percentage of pups with both eyes open; **p<0.01 vs. relevant control; *p<0.05 vs. relevant control; MA, methamphetamine. See text for details of statistical analyses.

Ano-Genital Distance

On PND 3, a significant effect of treatment was found (K=111.19, $p<0.001$) and a significant effect of sex was found (U=25.50, $p<0.001$) (Table 2.4) with females having a smaller ano-genital distance compared to males. A *post-hoc* test showed that on PND 3, for males the 2.5 mg/kg ($p<0.01$) group had a smaller ano-genital distance compared to the control male group. On PND 24, a significant effect of treatment was found (K=106.60, $p<0.001$) and a significant effect of sex was found (U=1.00, $p<0.001$) (Table 2.4) with females having a smaller ano-genital distance compared to males. A *post-hoc* test showed that there were no differences between the treatment groups compared to the control group for males or females.

Drug	PND 3 (mm)	PND 24 (mm)
Male		
Control	3.6 (3.1-4.0)	15.0 (14.0-16.1)
0.625 mg/kg	3.8 (3.4-4.3)	15.1 (13.9-16.3)
1.25 mg/kg	3.8 (3.4-4.0)	14.5 (13.4-16.6)
2.5 mg/kg	3.2 (2.8-3.5)**	14.7 (14.2-15.9)
Female		
Control	2.1 (1.8-2.2)	9.1 (8.7-9.9)
0.625 mg/kg	2.2 (1.9-2.50	9.3 (8.2-9.9)
1.25 mg/kg	2.2 (1.9-2.5)	9.0 (8.6-9.7)
2.5 mg/kg	1.9 (1.7-2.2)	9.4 (8.6-9.9)

Table 2.4: Rat pup ano-genital distance on PND 3 and 24. Ano-genital distance of male and female pups on PND 3 and 24 for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=17-21/group). Data are expressed as Median and

Interquartile range, $^{**}p<0.01$ vs. relevant control; MA, methamphetamine. See text for details of statistical analyses.

Body Length

No significant effect of sex was found on PND 7 ($U=1814.50$, $p>0.05$) or PND 14 ($U=2311.00$, $p>0.05$). No significant effect of treatment was found on PND 14 ($K=12.52$, $p>0.05$). A significant effect of treatment was found on PND 7 ($K=38.45$, $p<0.001$) (Figure 2.3 A and B). A *post-hoc* test showed that for males on PND 7 the 2.5 mg/kg MA group ($p<0.01$) had smaller body lengths compared to the control male group. A *post-hoc* test showed that for females on PND 7 the 2.5 mg/kg MA group ($p<0.05$) had smaller body lengths compared to the control female group. The 0.625 mg/kg MA group ($p<0.05$) for both males and females had larger body lengths compared to their control groups.

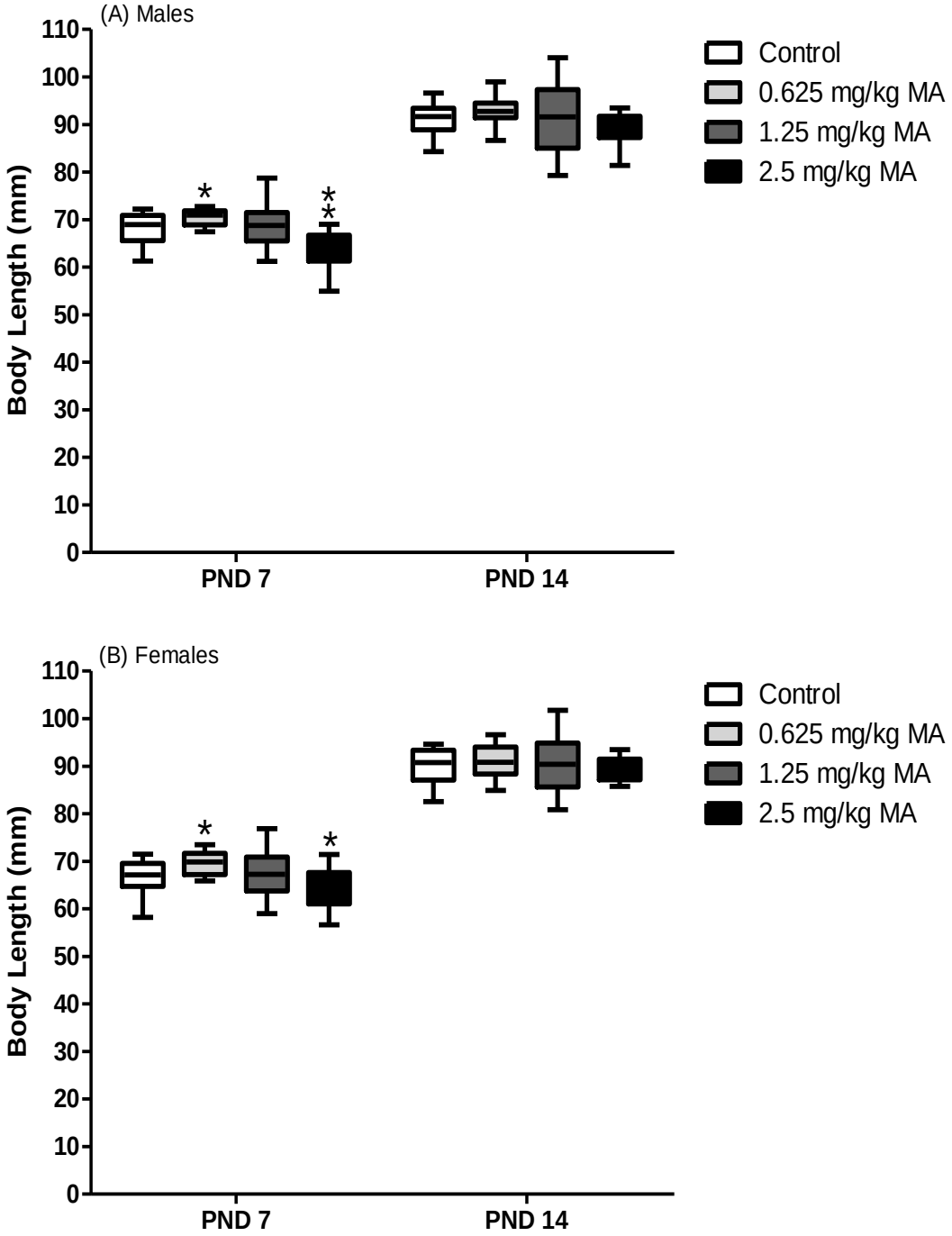

Figure 2.3: Rat pup body length on PND 7 and 14. Body Length for (A) male and (B) female pups for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=17-20/group). Data are expressed as Median, Interquartile range, min and max; **p<0.01 vs. relevant control; *p<0.05 vs. relevant control; MA, methamphetamine. See text for details of statistical analyses.

Neonatal Body Weight Gain

No significant effect of sex was found (U=175.00, p>0.05). No significant effect of treatment was found (K=12.10, p>0.05) (Figure 2.4).

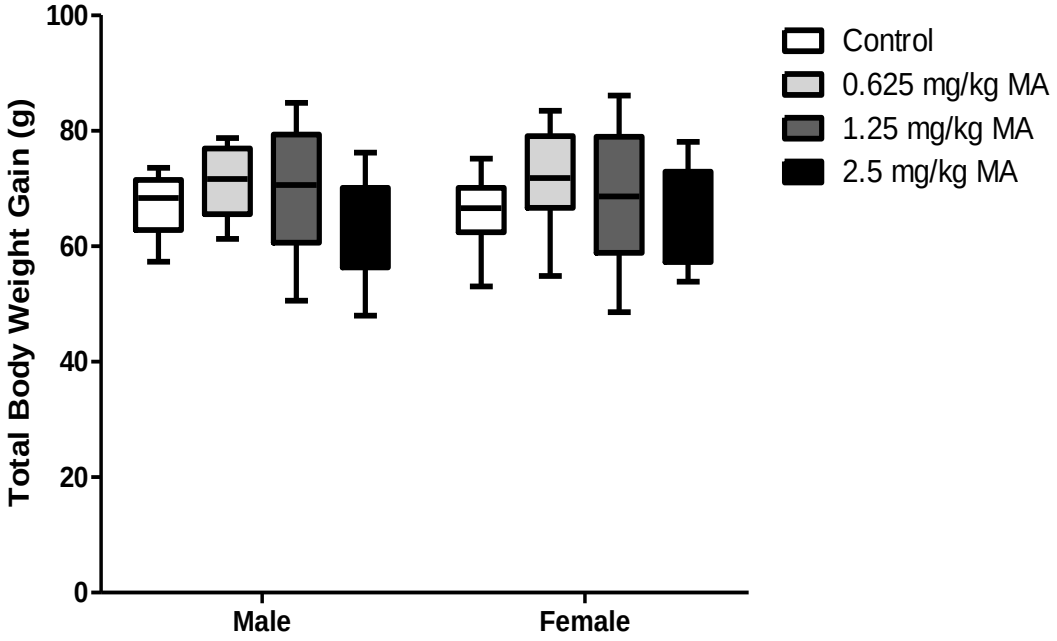

Figure 2.4: Neonatal total body weight gain. Total body weight gain in the neonatal period for male and female pups for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=17-20/group). Data are expressed as Median, Interquartile range, min and max; MA, methamphetamine. See text for details of statistical analyses.

2.3.3 Behavioural Testing of Offspring

Surface Righting

No significant effect of sex was found on PND 2 (X_1^2=2.00, p>0.05), PND 3 (X_1^2=0.09, p>0.05) or PND 4 (X_1^2=0.19, p>0.05). A significant effect of sex was found on and PND 5 (X_1^2=8.87, p<0.01) with more males righting themselves in less than 10 sec. A significant effect of treatment was found on PND 2 (X_1^2=62.22,

$p<0.001$) and PND 3 ($X_1^2=57.39$, $p<0.001$), PND 4 ($X_1^2=39.64$, $p<0.001$) and PND 5 ($X_1^2=42.49$, $p<0.001$) (Table 2.5). A *post-hoc* test showed that for males on PND 2 more pups in the 0.625 ($p<0.001$) and 2.5 mg/kg ($p<0.001$) MA groups could right themselves in less than 10 sec. On PND 3 in the 1.25 ($p<0.001$) and 2.5 mg/kg ($p<0.001$) MA groups and on PND 4 in the 1.25 mg/kg MA group ($p<0.05$) fewer pups could right themselves in less than 10 sec. A *post-hoc* test showed that for females on PND 2 more pups in the 0.625 mg/kg MA group ($p<0.05$) could right themselves in less than 10 sec. On PND 3 in the 2.5 mg/kg MA group ($p<0.001$), on PND 4 in the 1.25 ($p<0.05$) and 2.5 mg/kg ($p<0.001$) MA groups and on PND 5 in the 1.25 mg/kg MA group ($p<0.001$) fewer pups could right themselves in less than 10 sec.

Drug	PND 2 (%)	PND 3 (%)	PND 4 (%)	PND 5 (%)
Male				
Control	35	70	65	80
0.625 mg/kg	65***	75	70	85
1.25 mg/kg	33	44***	50*	89
2.5 mg/kg	70***	55***	60	70
Female				
Control	45	55	70	80
0.625 mg/kg	60*	75	80	80
1.25 mg/kg	33	50	56*	56***
2.5 mg/kg	45	40***	45***	70

Table 2.5: Rat pup surface righting. Surface righting for male and female pups on PND 2, 3, 4 and 5 for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=18-20/group). Data are expressed as percentage of pups that complete test in less than 10 sec, ***$p<0.001$ vs. relevant control; *$p<0.05$ vs. relevant control; MA, methamphetamine. See text for details of statistical analyses.

Air Righting

No significant effect of sex was found on PND 12 (U=2775.00, $p>0.05$) or PND 17 (U=2585.00, $p>0.05$). No significant effect of treatment was found on PND 12 (K=5.24, $p>0.05$) or PND 17 (K=13.10, $p>0.05$) (data not shown).

Negative Geotaxis

No significant effect of sex was found on PND 9 ($X_1^2=3.67$, $p>0.05$) or PND 11 ($X_1^2=0.68$, $p>0.05$). A significant effect of treatment was found on PND 9 ($X_1^2=32.40$, $p<0.001$) and PND 11 ($X_1^2=84.64$, $p<0.001$) (Figure 2.5). A *post-hoc* test showed that for males on PND 9 more pups in the 0.625 mg/kg MA group ($p<0.05$) could rotate themselves in less than 15 sec. On PND 11, more pups in the 1.25 mg/kg ($p<0.05$) and fewer pups in the 2.5 mg/kg ($p<0.001$) MA groups could rotate themselves in less than 15 sec. A *post-hoc* test showed that for females on PND 9 fewer pups in the 2.5 mg/kg MA group ($p<0.05$) could rotate themselves in less than 15 sec. On PND 11 more pups in the 0.625 mg/kg ($p<0.01$) and fewer pups in the 2.5 mg/kg ($p<0.001$) MA groups could rotate themselves in less than 15 sec.

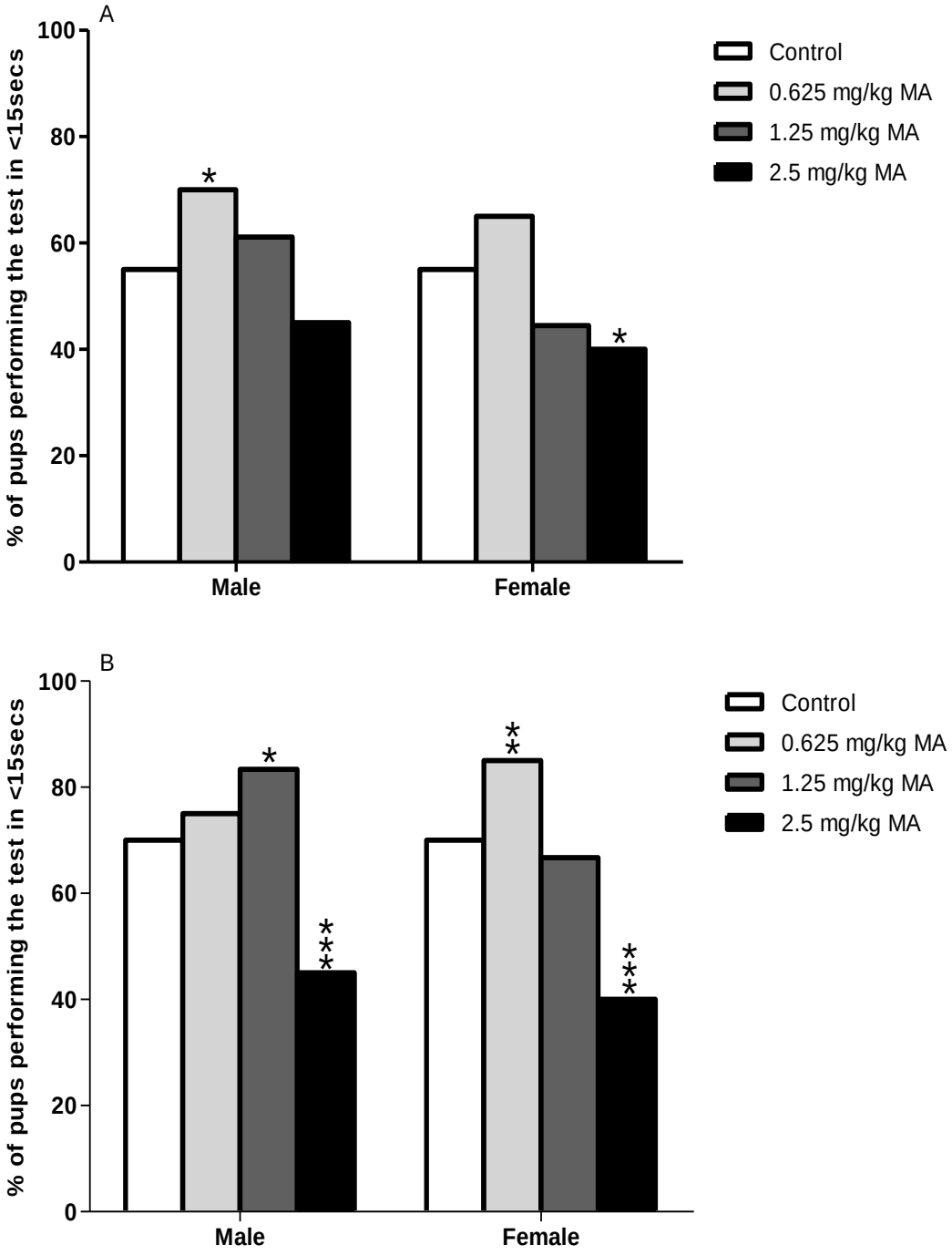

Figure 2.5: Rat pup negative geotaxis on PND 9 and 11. Negative Geotaxis for male and female pups on (A) PND 9 and (B) PND 11 for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=18-20/group). Data are expressed as percentage of pups that complete test in less than 15 sec; ***p<0.001 vs. relevant control; **p<0.01 vs. relevant control; *p<0.05 vs. relevant control; MA, methamphetamine. See text for details of statistical analyses.

Forelimb Grip

No significant effect of sex was found on PND 17 (X_1^2=2.38, p>0.05) and a significant effect of sex was found on PND 14 (X_1^2=12.29, p<0.001) with the females performing better on the forelimb grip test compared to the males. A significant effect of treatment was found on PND 14 (X_1^2=50.53, p<0.001) and PND 17 (X_1^2=26.14, p<0.001) (Figure 2.6). A *post-hoc* test showed that on PND 14 fewer pups in the 1.25 mg/kg MA group could perform the test for more than 10 sec for males (p<0.01) and females (p<0.001). On PND 17, for females fewer pups in the 1.25 mg/kg MA group (p<0.01) could perform the test for more than 10 sec.

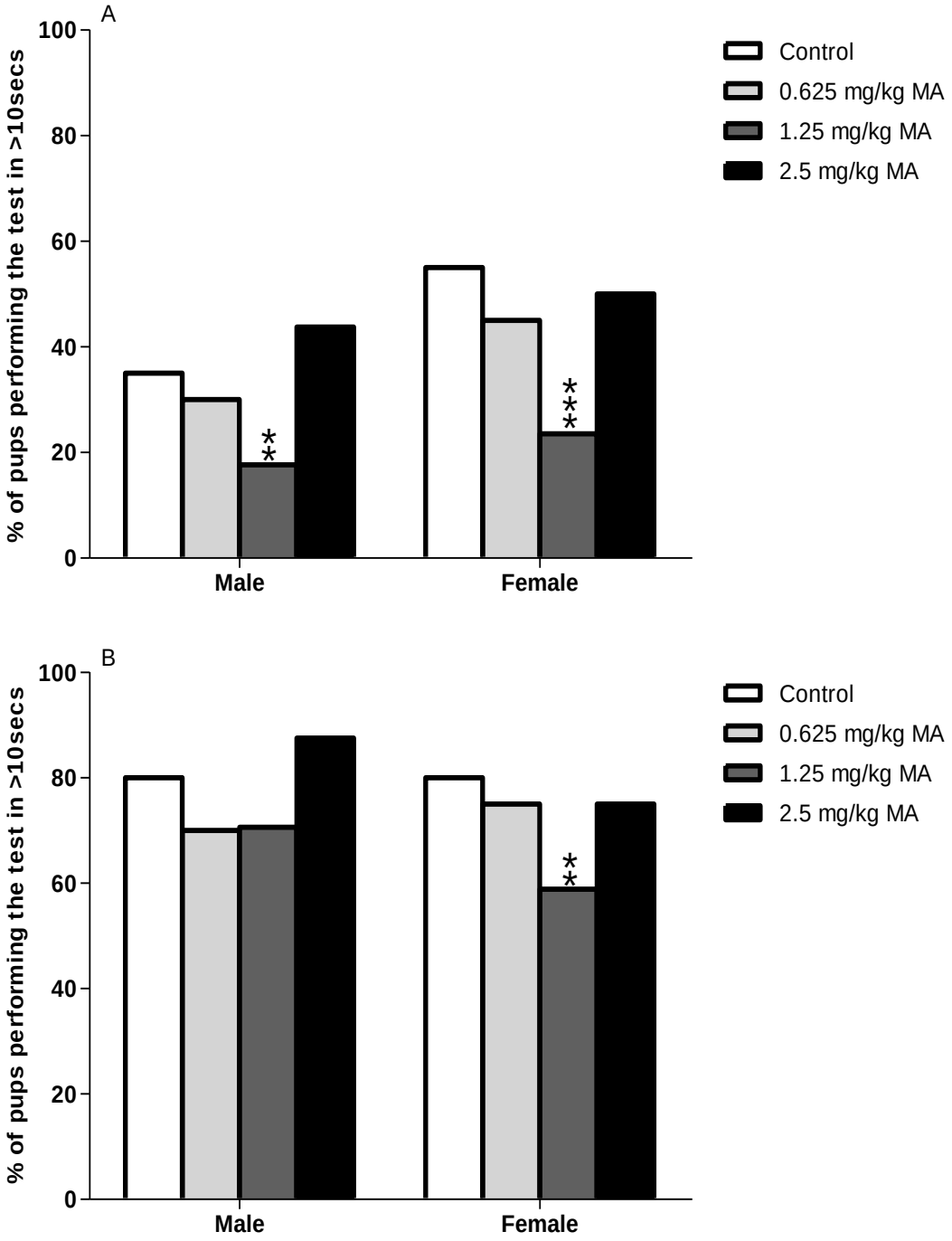

Figure 2.6: Rat pup forelimb grip on PND 14 and 17. Forelimb grip for male and female pups on (A) PND 14 and (B) PND 17 for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=16-20/group). Data are expressed as percentage of pups that complete test for more than 10 sec; ***p<0.001 vs. relevant control; **p<0.01 vs. relevant control; MA, methamphetamine. See text for details of statistical analyses.

Elevated Plus Maze and Open Field

In the EPM, no significant effect of sex was found for distance ($F_{(1, 133)}$=0.01, p>0.05), velocity ($F_{(1, 133)}$=0.02, p>0.05), open arm time (OAT) ($F_{(1, 133)}$=2.86, p>0.05) or open arm entries (OAE) ($F_{(1, 133)}$=0.01, p>0.05). No significant effect of treatment was found for OAT ($F_{(3, 133)}$= 1.13, p>0.05) or OAE ($F_{(3, 133)}$=1.58, p>0.05) (Table 2.6). No significant interaction effect of sex and treatment was found for distance, velocity, OAT or OAE. A significant effect of treatment was found for distance ($F_{(3, 133)}$=3.66, p<0.05) (Table 2.6) and velocity ($F_{(3, 133)}$=3.63, p<0.05) (data not shown). A *post-hoc* test showed that there were no differences between the treatment groups compared to the control group for distance or velocity.

In the OF, no significant effect of sex was found for distance ($F_{(1, 133)}$=0.09, p>0.05) or velocity ($F_{(1, 133)}$=0.10, p>0.05). No significant effect of treatment was found for distance ($F_{(3, 133)}$=0.21, p>0.05) (Table 2.6) and velocity ($F_{(3, 133)}$=0.21, p>0.05) (data not shown). No significant interaction effect of sex and treatment was found for distance or velocity.

Drug	OF	EPM		
	Distance Moved (cm)	Distance Moved (cm)	% Open Arm Time	% Open Arm Entries
Male				
Control	1968 ± 752	1644 ± 277	27 ± 15	32 ± 13
0.625 mg/kg	1806 ± 648	1572 ± 235	29 ± 15	37 ± 10
1.25 mg/kg	1872 ± 544	1447 ± 225	31 ± 12	37 ± 10
2.5 mg/kg	1781 ± 455	1540 ± 207	27 ± 16	33 ± 13
Female				
Control	1899 ± 400	1646 ± 311	23 ± 13	33 ± 12
0.625 mg/kg	1844 ± 454	1454 ± 267	27 ± 14	34 ± 12
1.25 mg/kg	1854 ± 559	1455 ± 240	27 ± 16	38 ± 12
2.5 mg/kg	1985 ± 700	1614 ± 229	21 ± 12	32 ± 12

Table 2.6: Elevated plus maze and open field on PND 29. Parameters of the EPM and OF for males and females for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=16-20/group). Data are expressed as Mean±SD; MA, methamphetamine. See text for details of statistical analyses.

Morris Water Maze

No significant effects of treatment or sex were found for time to find platform in the acquisition phase, or distance and velocity travelled, and time spent in the SW quadrant in the probe trial in the MWM (data not shown). A significant effect of treatment was found on entries into the SW quadrant in the probe trial ($F_{(13, 59)}$=4.61, p<0.01). A *post-hoc* test showed that for females the 0.625 mg/kg (p<0.05), 1.25 mg/kg (p<0.01) and 2.5 mg/kg (p<0.05) groups had less entries into the SW quadrant compared to the control female group (data not shown).

2.4 Discussion

The present study has revealed the consequences associated with MA abuse during pregnancy using a clinically relevant animal model. The findings show that prenatal MA exposure can have a negative influence on the neurodevelopment of the rat offspring.

The first aim of this study was to determine if prenatal MA exposure has any consequences on maternal parameters. There was no effect of MA treatment on weight gain, food consumption or water consumption. This is consistent with other studies that found no differences in maternal weight gain after MA exposure during the pre-mating, gestation and lactation periods using 5 mg/kg of MA, albeit using the subcutaneous route of administration (Pometlova *et al.*, 2009, Slamberova *et al.*, 2005a). MA is known to have anorectic properties (Kraeuchi *et al.*, 1985) and therefore mothers who abuse MA may gain less weight during pregnancy due to reduced food consumption. Other early preclinical studies with similar doses of MA (2, 3, 4.5 mg/kg and above) have shown these effects with maternal weight gain being suppressed compared to the controls (Cho *et al.*, 1991). From these findings it may be postulated that any deficits in offspring seen may be due to these anorectic effects and not solely due to the MA *in utero* exposure and this gives cause for concern with regard previous studies that have looked at extremely high doses of MA i.e. 80 mg/kg per day (Heller *et al.*, 2001). As these effects were not observed in this study then we can be confident in that any effects seen in the offspring are due to the MA treatment alone and not due to nutrition restrictions during the pregnancy or maternal toxicity.

Other factors to consider with regard to maternal parameters would be maternal care and behaviour. Some reports have suggested that maternal care can be distorted by prenatal drug use (Vavrinkova *et al.*, 2001) and that the mother–pup contact can be altered due to altered maternal behaviour (Bridges and Grimm, 1982). This in turn can contribute to indirect neurodevelopmental deficits in the offspring. Maternal behaviours include nursing, contact with pups, grooming and carrying pups, manipulating nest shavings, self-grooming, resting, rearing and sniffing (Slamberova *et al.*, 2005b). Slamberova *et al.* (2005a) demonstrated that MA exposure altered the blanket position of active nursing but there was no differences seen in other maternal activities. As our study did not look at maternal behaviour in the prenatal or postnatal period, then we can only hypothesise that by using these lower doses and also by not seeing any other changes in maternal parameters there was no behavioural alterations in the mothers.

Cross fostering has been employed in studies previously to try to overcome the limitations associated with altered maternal behaviour after drug exposure. This method has merit as all pups including controls are exposed to the same maternal care and any deficits found can be correlated to the *in utero* exposure to a drug and not to poor postnatal care of the mother. Previous studies have shown that pups exposed to MA prenatally and cross fostered to control mothers have a better performance in some sensorimotor tests and that this may be because the maternal care partially improves the impairing effect of MA (Pometlova *et al.*, 2009). However, when looking at memory and learning effects of prenatal MA exposure in the MWM, Hruba *et al.* (2009a) found that in adulthood all rats prenatally exposed to MA (5 mg/kg), regardless of postnatal exposure (MA or saline), exhibited deficits in

the MWM. This therefore shows that the changes in behaviour in the MWM after prenatal MA are still apparent whether MA or saline treated mother cares for the pups. Cross-fostering was not employed in this study as the risk exists that a prenatally exposed MA pup may indirectly expose the control mother to MA by urinary or faecal excretion as MA can still be present in their system, although to our knowledge this has not been previously investigated. Clinical studies have shown that MA is present in meconium samples of new-borns to MA-abusing mothers at birth (Arria *et al.*, 2006). This may then in turn alter the maternal behaviour during the early postnatal period. Even though evidence exists to show that in the retrieval test the first pup carried into the nest by the mother has no correlation to it being adopted or not (Slamberova *et al.*, 2005a), there is a risk of the maternal care being different towards their own pups and fostered pups. An observation time of 10 min on each PND may not be enough to accurately depict the overall maternal care towards fostered pups.

The second aim of this study was to determine if prenatal MA exposure has any consequences on neurodevelopment of the rat offspring. The present findings illustrate that MA can have a profound effect on the development of the pups with most of the results having been reported in the 2.5 mg/kg MA group. Delays in somatic development of the pups were evident in measures such as ano-genital distance, fur appearance, eye opening and body length. The sexual maturation of the males was significantly different in that the ano-genital distance was smaller in the 2.5 mg/kg group in comparison to the control. This is in disagreement with Slamberova *et al.* (2006) who did not find any difference in vaginal opening in females or testes descent in males at a higher dose of 5 mg/kg. However testes

descent was delayed after subcutaneous administration of MA from GD 7 to 20 at 3 mg/kg and not in the 1, 2 or 4.5 mg/kg groups in another study (Cho *et al.*, 1991). These masculising and feminising effects are an indication of developmental delay in the offspring. In the present study, these developmental delays were also apparent in fur appearance and eye opening. The 2.5 mg/kg MA group had less pups with fur for males and females and this delay was still apparent further into the neonatal period when the 2.5 mg/kg MA group had less pups with eyes open for females and males.

Interestingly, we found some contrasting results where prenatal MA at low doses enhanced the development of pups. The 1.25 mg/kg MA female group had more pups with eyes open in comparison to the control pups i.e. development occurred earlier. This phenomenon of earlier development was also evident in the 0.625 mg/kg MA group in parameters including surface righting, negative geotaxis and pinna unfolding in both males and females. Body length was taken as a measure of physical growth and maturation and this was also larger for the 0.625 mg/kg male and female groups; however, it was stunted for both male and female pups in the 2.5 mg/kg MA group when measured a week after birth. This has also been seen in clinical studies where children had low body length (Oei and Lui, 2007). In our study, however, the delay was not apparent one week later when the measurement was repeated suggesting that this impairment can be overcome. Again the body length can be correlated back to the neonatal body weight gain of the pups which was also decreased for males and females in the early postnatal period and not in the later postnatal period (after PND 11).

Delays in behavioural development of the pups were evident in measures such as surface righting, negative geotaxis and forelimb grip. Again, in the 2.5 mg/kg group fewer male and female pups could perform the righting reflex in less than 10 sec or rotate themselves in the negative geotaxis in less than 15 sec. Interestingly these deficits were also apparent for males and females in the 1.25 mg/kg MA group in the surface righting test, the negative geotaxis and in the forelimb grip where pups performed poorer compared to controls. This data are consistent with other reports that showed deficits in sensorimotor function and postural reflexes of offspring exposed to a higher dose of MA (5 mg/kg) (Hruba *et al.*, 2009b) yet it is in contrast to other earlier studies that showed no effect of prenatal amphetamine exposure at low doses similar to this study (Vorhees, 1985). The differences seen here may be due to the greater potency of MA when compared to amphetamine (NIDA Research Report, 2006) and therefore causing a more noticeable deficit in the behavioural development of the rat offspring. No significant deficits were found in the air righting reflex test. As expected, there were significant sex effects seen in parameters such as ano-genital distance, birth weights and body weights and other sex effects were also seen in eye opening, surface righting and forelimb grip. This highlights the importance of looking at both sexes and the possibility of neurodevelopment deficits being different between sexes.

We did not see a significant difference on the parameters within the EPM or OF and to our knowledge, no studies have looked at these parameters in the adolescent period. No significant results were found in the MWM. Due to these findings, future work would aim to explore other tests and assessments to see if the delayed

development persists and if prenatal MA has an effect in later life when the offspring reach adulthood.

2.5 Conclusions

The present findings have shown that by using pharmacologically relevant doses of MA and a preclinical model that has many similarities to the clinical situation, MA can have a profound dose-related effect on neonatal outcome. The doses used within this study are within the range (when extrapolated to humans) that are commonly abused by pregnant females and if extrapolated to the clinical scenario this will give cause for concern regarding the risks associated with this drug of abuse at relatively low doses. Again, the consequences of these retardations in later life is of concern and the question of whether or not these effects can disappear and be rectified over time, however further work is needed to elucidate this.

Declaration

The following work has not been published previously, is not under consideration for publication elsewhere and its publication is approved by all authors. If accepted, it will not be published elsewhere including electronically, in English or in any other language, without the written consent of the copyright-holder.

Disclosure

The authors have no conflict of interest.

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Chapter 3:

The consequences of prenatal and/or postnatal methamphetamine exposure on neonatal development and behaviour in rat offspring

McDonnell-Dowling, K. & Kelly, J. P. 2015. The consequences of prenatal and/or postnatal methamphetamine exposure on neonatal development and behaviour in rat offspring. *International Journal of Developmental Neuroscience,* 47, Part B**,** 147-156.

Abstract

Methamphetamine has become a popular drug of abuse in recent years not only in the general population but also amongst pregnant women. Although there is a growing body of preclinical investigations of MA exposure during pregnancy, there has been little investigation of the consequences of such exposure via the breast milk during the neonatal period. Therefore, the aim of this study was to determine the consequences of MA exposure during pregnancy and/or lactation on neurodevelopment and behaviour in the rat offspring. Pregnant Sprague-Dawley dams received MA (3.75 mg/kg) or control (distilled water) once daily via oral gavage from gestation day 7-21, postnatal day 1-21 or gestation day 7- postnatal day 21. A range of well-recognised neurodevelopmental parameters were examined in the offspring. Prenatal MA significantly reduced maternal weight gain, with a concomitant reduction in food intake. A significant increase in neonatal pup mortality was observed, being most marked in the prenatal/postnatal MA group. Significant impairments in neurodevelopmental parameters were also evident in all MA treatment groups including somatic development (e.g. pinna unfolding, fur appearance, eye opening) and behavioural development (e.g. surface righting, inclined plane test, forelimb grip). In conclusion, this study demonstrates that exposure to MA during any of these exposure periods (prenatal and/or postnatal) can have a profound effect on neonatal outcome, suggesting that regardless of the exposure period MA is associated with detrimental consequences in the offspring. These results indicate that in the clinical scenario, exposure during lactation needs to be considered when assessing the potential harmful effects of MA on offspring development.

3.1 Introduction

The use of MA has had a surge in popularity in recent years and MA is presently the most widely used illicit drug after cannabis (National Drug & Alcohol Research Centre, 2007, United Nations Office on Drugs and Crime, 2013). In 2012, the average age at first use of MA was 19.7 years old (Substance Abuse and Mental Health Services Administration, 2013). This age can be considered within the 'child bearing years' and consequently a time when females may become pregnant while using MA (Anderson and Choonara, 2007). Although preclinical and clinical literature exists documenting the adverse effects of this drug on offspring, there remains a significant amount of pregnant females abusing MA with one study showing a 5.2% prevalence in areas of America where MA abuse has become a problem (Arria *et al.*, 2006).

Prenatal MA exposure has been extensively studied preclinically (Slamberova *et al.*, 2005b, Slamberova *et al.*, 2008, Schutova *et al.*, 2010, Hruba *et al.*, 2010, McDonnell-Dowling *et al.*, 2014) however, the risks associated with postnatal exposure is something that has been overlooked in the preclinical literature. It has been established that MA can pass easily from the mother to infant via the breast milk (Bartu *et al.*, 2009) and that some mothers may increase their use of amphetamines after birth and in the first few months of breastfeeding (Bartu *et al.*, 2006). As highlighted in our recent review (McDonnell-Dowling and Kelly, 2015) there are a dearth of preclinical studies examining the effects of MA on the offspring when exposed via the breast milk of the mother. It is unknown if the effects of MA exposure during breastfeeding is comparable to the harmful effects of MA exposure during pregnancy. Recent work by Rambousek *et al.* (2014) demonstrated that the plasma concentrations of MA in pups at birth after exposure during gestation are

greater than plasma concentrations of MA in pups at weaning after exposure during lactation. Therefore it is unknown if the risk for these offspring or the consequences on these offspring is greater if exposed to MA during gestation compared to lactation.

Chasnoff (1987) reported that following abstinence from cocaine during pregnancy one mother admitted her two week old infant to hospital with clinical manifestations of cocaine intoxication (hypertension, irritability, tachycardia and tremulousness) after she consumed cocaine during breastfeeding. If a mother remains abstinent from MA during her pregnancy but then relapses during the breastfeeding period the effects on the developing infant are largely unknown. Therefore, the aim of this study was to determine if MA exposure during pregnancy and/or lactation at a pharmacological dose affects neurodevelopment and behaviour in the rat offspring. The MA dose employed should represent the clinical scenario and selection was aided by our previous dose response study (McDonnell-Dowling *et al.*, 2014). We employed a novel route of administration, as to the best of our knowledge oral gavage has never been employed in preclinical studies. The hypothesis is that both prenatal and postnatal MA exposure at a pharmacological dose when given orally will have an adverse effect on the rat offspring.

3.2 Materials and Methods

3.2.1 Animal Housing

Adult male (275–325 g, approx. 4 months old) and female (275–325 g, approx. 4 months old) Sprague-Dawley rats were used for this study. All females were bred in house, all males were obtained from Charles River (Kent, U.K.) and animals were habituated for one week from arrival. After mating, all female rats were housed

singly in plastic bottom cages with additional nesting materials. All animals were maintained under standard laboratory conditions under artificial 12-h light/dark cycle (lights on from 08:00h) and temperature was maintained at 20-24^{o}C with relative humidity at 35-60%. Food and water were provided *ad libitum*. Following littering, the rat pups remained with their biological dams until PND 21, at which point the pups were weaned. Cross-fostering was not employed in this study in order to mimic the clinical scenario but also to ensure that active drugs present in the MA mother are not passed onto the control pups via breastmilk or urinary and faecal excretions (McDonnell-Dowling and Kelly, 2015c). All experiments were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway (12/NOV/07) and in compliance with the European Communities Council directive 86/609.

3.2.2 Mating

For this study, 52 female rats and 12 male rats were used. A male:female ratio of 1:3 for mating was used. Each cage of female rats (three) was housed overnight with one sexually mature male rat. At the beginning of the light phase the following morning, vaginal smears were obtained from all females to check for the presence of sperm. All smears were examined under a light microscope. Gestation day 0 was deemed the day that sperm was present in the smear. Of the 52 females that were mated with males, 37 females became pregnant (71% success rate).

3.2.3 Gestation Period and Deliveries

The pregnant females were checked daily and the expected day of delivery (birth) in rats is GD 21-22 (Daston *et al.*, 2004). Offspring in each litter were checked and

counted daily in the week after delivery to monitor for pup mortalities. The pups were randomly culled (using a random number generator) to 10 per litter on PND 1 with a litter ratio of 50:50 males:females whenever possible. One male and one female were selected for testing from each litter in order to avoid litter effects and these same pups continued through all neonatal testing. These two pups were selected as they represented the average weight of the litter. Each of these pups was injected intradermally with black, India ink in the footpad for unique identification purposes in a litter.

3.2.4 Drug Treatment

Methamphetamine HCl was purchased from Sigma Aldrich (Wicklow, Ireland; M8750). Rats were assigned randomly to control or MA treated groups based on body weight and likelihood of pregnancy. The dose of MA given was 3.75 mg/kg at a volume of 1 ml/kg and controls received the vehicle alone (VEH), i.e. 1 ml/kg distilled water. Our previous dose response study (McDonnell-Dowling *et al.*, 2014) aided in deciding this dose as it relates to a clinically relevant dose in the human scenario and the use of an allometric scale takes into account the body weight and body surface area of a pregnant human versus a pregnant rat (Reagan-Shaw *et al.*, 2008) therefore maintaining the viability of the project and its comparison to clinical situations. For MA or control treatments, the dams were dosed via oral gavage once daily at 14.00h from GD 7 until PND 21 (time of weaning). Oral gavage was used as this represents the most common route of MA administration in humans (U.S. Department of Health and Human Services, 2006), but has heretofore been disregarded in preclinical investigations. During the gestation period two groups of dams received VEH and two groups received MA. During the postnatal period, one

group of dams receiving VEH continued to take VEH and the other was switched to MA, one group of dams receiving MA continued to take MA and the other was switched to VEH. This gave four treatment groups.

3.2.5 Maternal Daily Measurements

Maternal body weight was recorded daily from GD 0 to PND 21 prior to dosing of each rat (between 14.00 and 16.00h). Maternal food and water consumption were also recorded daily from GD 0. Although maternal behaviours were not examined in this study, previous work in our lab has shown that when given orally at a dose of 3.75 mg/kg MA does not impair maternal behaviour in the observation or retrieval test (unpublished data). On PND 21 (day of weaning), all dams were sacrificed by decapitation.

3.2.6 Development of Offspring

The development of the offspring involved examining somatic development and behavioural testing. The day on which each test was performed related to the time at which its development milestone normally occurs in rats and each test has a specific PND. Both dam and pups remained in the home cage room while testing occurred. At the time of testing, the dams were removed from the home cage and placed in a separate cage. The pups were taken directly from the home cage and placed back into the home cage after testing was completed.

Somatic Development

Somatic parameters included pinna (ear) unfolding, fur apperance, eye opening, ano-genital distance, body lengths and body weights. Pinna unfolding was recorded from

PND 3, eye opening was recorded from PND 14 and fur appearance was recorded from PND 3 for males and females. The time of first appearance of fur was considered the first day of occurrence, whilst in the cases of pinna unfolding and eye opening, where both pinna or eyes must unfold or open respectively to denote the first day of appearance. Recording of these parameters continued until all pinna had unfolded, eyes had opened and fur was present in all rat pups.

Ano-Genital Distance

Ano-genital distance was measured (for comparison) for possible masculinising or feminising effects of MA. This was measured using a digital calipers between the base of the genitals and the top of the anus. This was performed on PND 3.

Body Length

Body length was measured for each pup to compare growth sizes. This was measured using a digital calipers between the tip of the nose and the base of the tail. This was performed on PND 7 and 14.

Body Weight

Body weight was measured for each pup throughout the neonatal period on PND 1, 2, 4, 8, 11, 15 and 18 prior to behavioural testing each day.

Behavioural Testing

Righting Reflex on a Surface

Pups were placed in the supine position on a flat surface and the time taken to turn over and restore its normal prone position (on all fours) was recorded. The maximum time allowed was 30 s. A time of 30 s was recorded if the pup did not right itself within this period and the test was terminated. This test was performed on PND 2, 3, 4 and 5.

Inclined plane

The inclined plane apparatus consists of a flat timber surface that is inclined and held at a 30˚ angle. The pups were placed facing downward at the top of the inclined plane apparatus. The time taken to turn 180˚ and face upward was recorded. The maximum time allowed was 30 s. A time of 30 s was recorded if the pup did not turn within this period and the test was terminated. This test was performed on PND 9 and 11.

Forelimb Grip

The forelimb grip apparatus consists of a thin steel bar supported by two adjustable poles. The bar is approx. 20 cm in length, 0.2 cm in diameter and lies 25 cm above the base of the platform. The pup was gripped at the base of the tail and lowered onto the bar. The length of time the pup was able to hold on to the bar before falling was recorded and the maximum time allowed was 30 s. A time of 30 s was given if the pup did not fall within this period and the test was terminated. This test was performed on PND 14 and 17.

3.2.7 Statistical Analysis

All figures representing the data from the testing period were constructed using Graphpad Prism 5.0. The data were then analysed using the statistical package SPSS 20.0. Data were assessed initially to determine if it displayed normality of distribution and homogeneity of variance (Shapiro–Wilks and Levene's test $p>0.05$). This determines whether the data are parametric or non-parametric. The data were also assessed to determine if it displayed sphericity (Mauchly's test $p>0.05$) however if this failed then the Greenhouse–Geisser correction was used (i.e. degrees of freedom corrected). For the parametric data tests used included; repeated measures

ANOVA to compare the overall effect for related data, Two-Way ANOVA to compare the effect of treatment groups and sex, One-Way ANOVA and Student-Newman Keuls *post-hoc* tests were used to define where the significance lay. For the non-parametric data tests used included: Friedman's ANOVA by Ranks to compare the overall effect for related data, Wilcoxon Match-Pairs test to compare the effect of time, Kruskal-Wallis test to compare the effect of treatment groups and Mann-Whitney U tests were used to define where the significance lay. For pinna unfolding, eye opening and fur appearance the data were taken as counts (present or absent) and for surface righting, inclined plane test and forelimb grip the data were taken as counts (ability to perform the test or not) and so the Chi-Squared test was performed for these parameters. All results reported are for the MA treatment groups compared to the control group. The level of significance was set at $p<0.05$ for all parameters except for the Chi-Squared test which was set at $p<0.01$ (Bonferroni correction employed due to multiple comparisons).

3.3 Results

3.3.1 Prenatal Maternal Daily Measurements

Prenatal Maternal Weight Gain

A significant effect of gestation day was found ($F_{(2.18, 71.97)}=783.77$, $p<0.001$) with all groups gaining weight as gestation progressed. A significant interaction effect of gestation day and treatment was found ($F_{(6.54, 71.97)}=12.81$, $p<0.001$). A significant effect of treatment was found ($F_{(3, 33)}=3.16$, $p<0.05$) (Figure 3.1) with the MA/VEH group weighing less on GD 9-20 and the MA/MA group weighing less on GD 18-20. A significant effect of treatment was found for total body weight gain in the prenatal

period ($F_{(3, 33)}$=11.40, p<0.001). A *post-hoc* test revealed that the MA/VEH and MA/MA groups gained less weight in the prenatal period compared to the VEH/VEH group (data not shown).

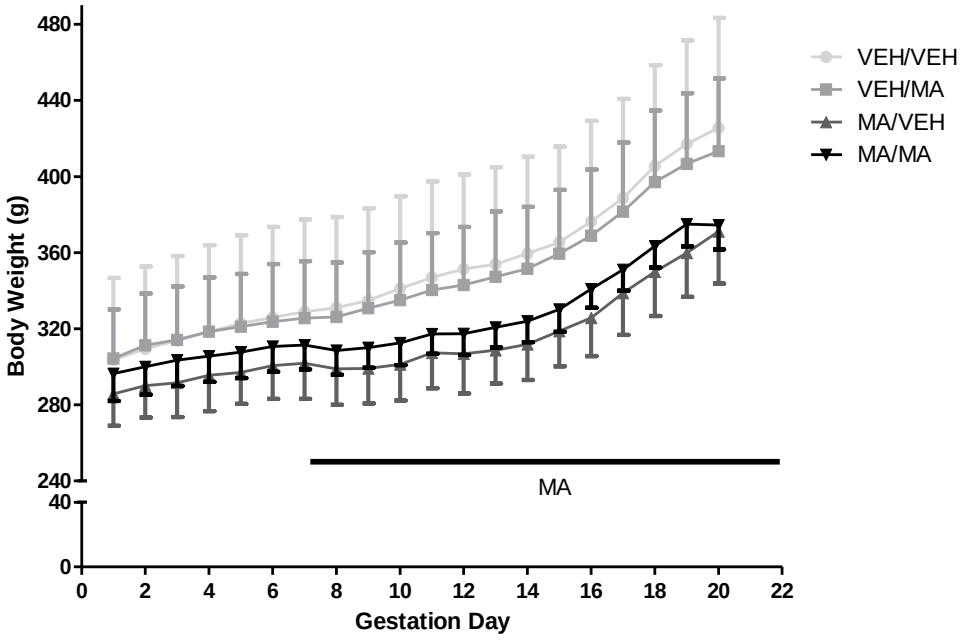

Figure 3.1: Maternal weight gain during gestation. Weight gain for each day of gestation (n=8-10/group). Line indicates dosing period. Data are expressed as Mean±SD.

Prenatal Maternal Food Consumption

A significant effect of gestation day was found ($F_{(8.35, 267.07)}$=15.92, p<0.001) with food consumption increasing slightly throughout the gestation period and then decreasing for the last few days preceding birth (GD 18 to 21). A significant interaction effect of gestation day and treatment was found ($F_{(25.04, 267.07)}$=2.73, p<0.001). A significant effect of treatment was found ($F_{(3, 32)}$=8.01, p<0.001) (Figure 3.2). A *post-hoc* test showed that the MA/VEH and MA/MA groups consumed less

food compared to the VEH/VEH group on GD 7-18. No significant effect of treatment was found for total food consumption in the prenatal period (data not shown).

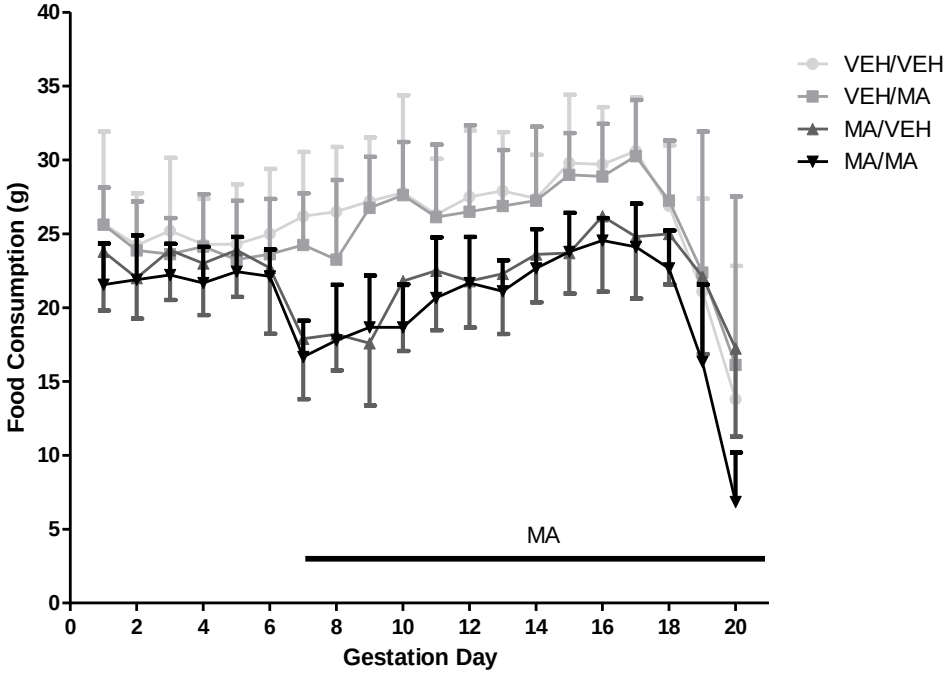

Figure 3.2: Maternal food consumption during gestation. Food consumption for each day of gestation (n=8-10/group). Line indicates dosing period. Data are expressed as Mean±SD.

Prenatal Maternal Water Consumption

A significant effect of gestation day was found ($F_{(4.30,\ 133.24)}$=21.00, p<0.001) with water consumption increasing slightly throughout the gestation period and then decreasing for the last few days preceding birth (GD 18 to 21). A significant interaction effect of gestation day and treatment was found ($F_{(12.89,\ 133.24)}$=2.06, p<0.05). No significant effect of treatment was found. No significant effect of

treatment was found for total water consumption in the prenatal period (data not shown).

3.3.2 Maternal and Litter Observations

No significant effect of treatment was found for day of birth/length of gestation period with all groups having a gestation length of 22 days. No significant effect of treatment was found for number of pups in a litter or number of males and females in a litter (data not shown). Maternal death did not occur in any treatment groups. No significant effect of treatment was found for percentage of stillborn pups. A significant effect of treatment was found for percentage of pups eaten in the postnatal period ($K=12.14$, $p<0.01$) in that the VEH/MA and MA/MA groups had a higher percentage of pups eaten compared to the VEH/VEH group. A significant effect of treatment was found for percentage of pups that died in postnatal period ($K=21.91$, $p<0.001$) in that the MA/MA group had a higher percentage of pups that died compared to the VEH/VEH group. A significant effect of treatment was found for total percentage of pup deaths in the postnatal period ($K=18.17$, $p<0.001$) in that the MA/MA group had a higher total percentage of pup deaths compared to the VEH/VEH group (Table 3.1). Based on the high percentage of pup death in the MA/MA group only four viable litters remained for testing in the postnatal period. Although the number of litters for this group was small, results were still included for comparison. The final number of litters used for testing was ten VEH/VEH litters, eight VEH/MA litters, ten MA/VEH litters and four MA/MA litters. The final number of litters used for testing was therefore 32.

Group	No. of Pups/Litter	% Pups Stillborn	% Pups Found Dead	% Pups Eaten	% Total Deaths
VEH/VEH	14 ± 2	4.1	0.0	0.8	5.0
VEH/MA	13 ± 4	0.8	2.1	15.5*	18.3
MA/VEH	12 ± 3	3.1	2.3	5.8	11.2
MA/MA	13 ± 2	5.5	32.4***	30.9**	68.7***

Table 3.1: Litter observations. Litter observations at birth and in the postnatal period (n=8-10 dams/group). Data are expressed as Mean±SD or % of pups; ***p<0.001, **p<0.01, *p<0.05 vs. VEH/VEH.

3.3.3 Postnatal Maternal Daily Measurements

Postnatal Maternal Weight Gain

A significant effect of postnatal day was found ($F_{(3.74, 82.22)}$=34.11, p<0.001) with all groups gaining weight as time progressed. A significant interaction effect of postnatal day and treatment was found ($F_{(11.21, 82.22)}$=2.13, p<0.05). No significant effect of treatment was found (Figure 3.3). Although it appears that the VEH/MA group gained less weight in the postnatal period, no significant effect of treatment was found for total body weight gain in the postnatal period (data not shown).

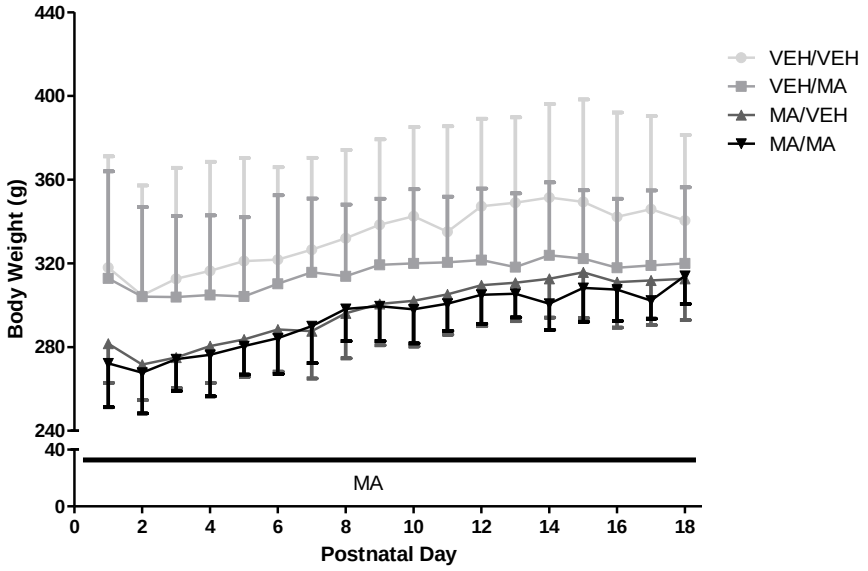

Figure 3.3: Maternal weight gain during lactation. Weight gain for each day of lactation (n=4-10/group). Line indicates dosing period. Data are expressed as Mean±SD.

Postnatal Maternal Food Consumption

No significant effect of postnatal day, treatment or interaction effect of postnatal day and treatment was found (Figure 3.4). A significant effect of treatment was found for total food consumption in the postnatal period ($F_{(3, 28)}$=4.12, p<0.05). A *post-hoc* test showed that the MA/MA group consumed less food compared to the VEH/VEH group (data not shown).

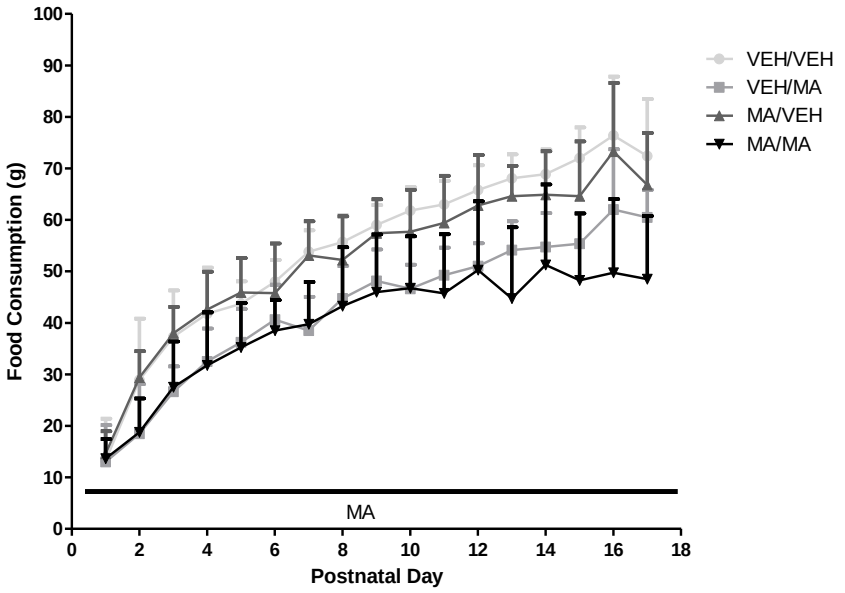

Figure 3.4: Maternal food consumption during lactation. Food consumption for each day of lactation (n=4-10/group). Line indicates dosing period. Data are expressed as Mean±SD.

Postnatal Maternal Water Consumption

A significant effect of postnatal day was found ($F_{(3.45,\ 72.44)}$=30.36, p<0.001). No significant effect of treatment or interaction effect of postnatal day and treatment was found. No significant effect of treatment was found for total water consumption in the postnatal period (data not shown).

3.3.4 Somatic Development of Offspring

Birth Weights

No significant effect of sex or interaction effect of treatment and sex was found. No significant effect of treatment was found (data not shown).

Pinna Unfolding

A significant effect of sex was found on PND 3 (X_1^2=3.92, p<0.05) with more males having eyes open than females and on PND 4 (X_1^2=10.53, p<0.01) with more females having eyes open than males. A significant effect of treatment was found on PND 3 (X_1^2=36.66, p<0.001), PND 4 (X_1^2=50.39, p<0.001) and PND 5 (X_1^2=91.59, p<0.001) (Table 3.2). A *post-hoc* test showed that on PND 3, for males the MA/MA group had less pups with pinna unfolded compared to their controls. On PND 4, for males and females, all MA groups had less pups with pinna unfolded compared to their controls. On PND 5, for males, the VEH/MA group had less pups with pinna unfolded compared to the control (data not shown).

Drug	PND 3 (%)	PND 4 (%)
Male		
VEH/VEH	20	90
VEH/MA	13	75**
MA/VEH	10	80
MA/MA	0***	67***
Female		
VEH/VEH	10	100
VEH/MA	13	75***
MA/VEH	10	70***
MA/MA	0	75***

Table 3.2: Pinna unfolding. Percentage of pups with pinna unfolded for male and female pups (n=4-10/group). Data are expressed as percentage of pups with both pinna unfolded; ***p<0.001, **p<0.01 vs. relevant control.

Fur Appearance

No significant effect of sex was found on PND 3 or 4. A significant effect of treatment was found on PND 3 (X_1^2=105.78, p<0.001) but not on PND 4 (Figure 3.5). A *post-hoc* test showed that, for males, the MA/VEH and MA/MA groups had less pups with fur compared to the control. For females, the MA/VEH group had less pups with fur compared to the control.

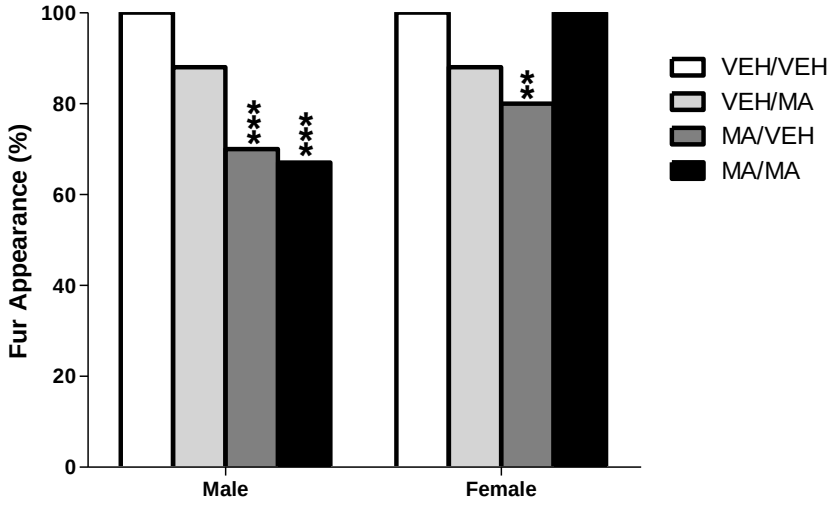

Figure 3.5: Fur appearance. Percentage of pups with fur for male and female pups on PND 3 (n=4-10/group). Data are expressed as percentage of pups with fur; ***p<0.001, **p<0.01 vs. relevant control.

Eye Opening

A significant effect of sex was found on PND 15 (X_1^2=38.10, p<0.001) with more females having eyes open than males but this was not seen on PND 14 or 16. A significant effect of treatment was found on PND 14 (X_1^2=86.92, p<0.001), PND 15 (X_1^2=80.64, p<0.001) and PND 16 (X_1^2=48.63, p<0.001) (Table 3.3). A *post-hoc* test showed that, for males, on PND 14 for the MA/MA group and on PND 15 for

the MA/VEH group, more pups had eyes open compared to their controls. For males on PND 15, the MA/MA group had less pups with eyes open compared to their controls. For females, on PND 14 for the MA/VEH group more pups had eyes open compared to their controls. On PND 15 and 16, the MA/MA group had less pups with eyes open compared to their controls. On PND 15, the VEH/MA and MA/VEH groups had less pups with eyes open compared to their controls.

Drug	PND 14 (%)	PND 15 (%)	PND 16 (%)
Male			
VEH/VEH	10	50	90
VEH/MA	0	63	88
MA/VEH	20	70**	90
MA/MA	33***	33**	100
Female			
VEH/VEH	10	90	90
VEH/MA	13	63***	88
MA/VEH	30***	60***	100
MA/MA	0	50***	75**

Table 3.3: Eye opening. Percentage of pups with eyes open for male and female pups (n=4-10/group). Data are expressed as percentage of pups with eyes open; ***p<0.001, **p<0.01 vs. relevant control.

Ano-Genital Distance

A significant effect of sex was found ($F_{(1, 55)}$=169.29, p<0.001) with females having a smaller ano-genital distance compared to males. No significant effect of treatment or interaction effect of sex and treatment was found (data not shown).

Body Length

No significant effect of sex or interaction effect of sex and treatment was found on any of the testing days. A significant effect of treatment was found on PND 7 ($F_{(3, 55)}$=4.07, p<0.05) but not on PND 14. A *post-hoc* test showed that there was no significant difference between treatment groups for males or females compared to the controls (data not shown).

Neonatal Body Weight

A significant effect of postnatal day was found ($F_{(2.04, 103.93)}$=953.71, p<0.001) with all groups gaining weight as time progressed. No significant effect of treatment, sex or interaction effect of sex and treatment was found (data not shown).

Neonatal Body Weight Gain

No significant effect of sex or interaction effect of sex and treatment was found for body weight gain (PND 1-18). A significant effect of treatment was found ($F_{(3, 55)}$=6.27, p<0.01) (data not shown). A *post-hoc* test showed that there was no significant difference between treatment groups for males or females compared to the controls.

3.3.5 Behavioural Testing of Offspring

Surface Righting

A significant effect of sex was found on PND 2 (X_1^2=9.52, p<0.01) with females performing better and on PND 3 (X_1^2=35.29, p<0.001) and PND 4 (X_1^2=22.22, p<0.001) with males performing better. A significant effect of treatment was found on PND 2 (X_1^2=197.02, p<0.001), PND 3 (X_1^2=296.52, p<0.001), PND 4

(X_1^2=259.87, p<0.001) and PND 5 (X_1^2=112.49, p<0.001) (Table 3.4). A *post-hoc* test showed that for females, the MA/MA group on each testing day, the VEH/MA group on PND 2, and the MA/VEH group on PND 3 and 5 had fewer pups that could reach the criterion of 10 s compared to the VEH/VEH group. For males, the MA/MA group on PND 3, 4 and 5, the VEH/MA group on PND 3, and the MA/VEH group on PND 3 and 4 had fewer pups that could reach the criterion of 10 s compared to the VEH/VEH group.

Drug	PND 2 (%)	PND 3 (%)	PND 4 (%)	PND 5 (%)
Male				
VEH/VEH	60	100	100	90
VEH/MA	75**	63***	100	88
MA/VEH	60	50***	80***	100
MA/MA	67	0***	33***	67***
Female				
VEH/VEH	80	70	80	90
VEH/MA	50***	88**	75	100
MA/VEH	80	50**	70	60***
MA/MA	0***	25***	25***	75**

Table 3.4: Surface righting. Surface righting for male and female pups on PND 2, 3, 4 and 5 (n=4-10/group). Data are expressed as percentage of pups that complete the test in less than 10 s; ***p<0.001, **p<0.01 vs. relevant control.

Inclined Plane

No significant effect of sex was found on PND 9 or 11. A significant effect of treatment was found on PND 9 (X_1^2=60.45, p<0.001) and PND 11 (X_1^2=148.14, p<0.001). For males on PND 11, the VEH/MA group had more pups that could reach the criterion of 15 s compared to the VEH/VEH group (Figure 3.6). For females, on

PND 9 the VEH/MA and MA/VEH groups, had more pups that could reach the criterion of 15 s compared to the VEH/VEH group (data not shown). On PND 11 the male and female MA/MA groups, had less pups that could reach the criterion of 15 s compared to the VEH/VEH groups (Figure 3.6).

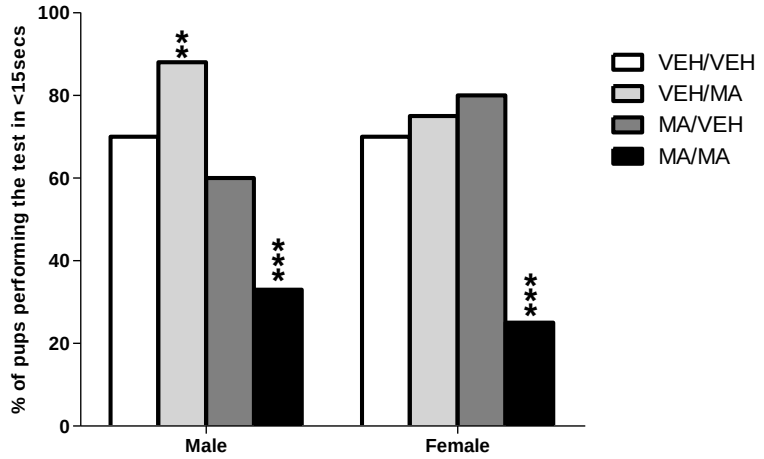

Figure 3.6: Inclined plane. Inclined plane test for male and female pups on PND 11 (n=4-10/group). Data are expressed as percentage of pups that complete test in less than 15 s; ***p<0.001, **p<0.01 vs. relevant control.

Forelimb Grip

A significant effect of sex was found on PND 14 (X_1^2=9.52, p<0.01) with males performing better than females and on PND 17 (X_1^2=8.00, p<0.01) with females performing better than males. A significant effect of treatment was found on PND 14 (X_1^2=108.15, p<0.001) and PND 17 (X_1^2=75.21, p<0.001) A *post-hoc* test showed that on PND 14, for males, the VEH/MA group had less pups that could perform the task for more than 10 s compared to the VEH/VEH group. For females on PND 14, for the VEH/MA group had less pups could perform the task and the MA/VEH group had more pups could perform the task compared to the VEH/VEH group

(Figure 3.7). On PND 17, the VEH/MA and MA/VEH groups more pups could perform the task for more than 10 s compared to the VEH/VEH group (data not shown).

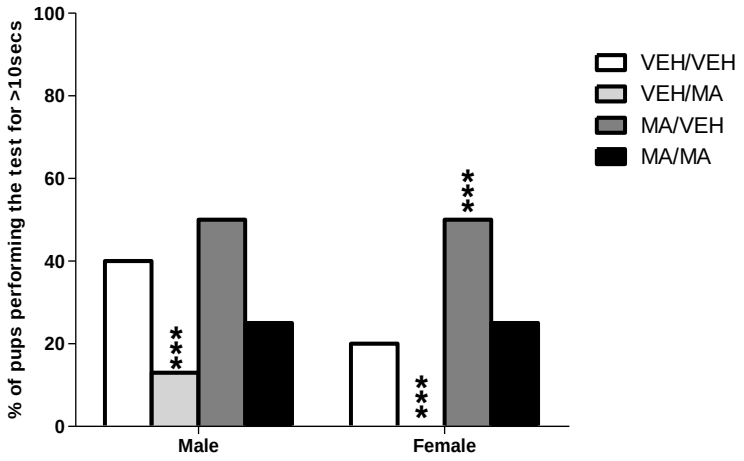

Figure 3.7: Forelimb grip. Forelimb grip for male and female pups on PND 14 (n=4-10/group). Data are expressed as percentage of pups that could perform the task for more than 10 s; ***p<0.001 vs. relevant control.

3.4 Discussion

The present study confirms that prenatal and/or postnatal MA exposure at a pharmacological dose has adverse effects on the developing offspring. MA exposure whether given prenatally and/or postnatally can delay neurodevelopment and alter behaviour in the rat offspring.

With regard to effects of MA exposure on the dams, MA administered prenatally at the present dose reduces body weight gain and food intake in the first days of drug administration. Similarly, when administered postnatally MA reduces body weight gain and food intake. MA is known to suppress appetite (Kraeuchi *et al.*, 1985) and although there is limited literature regarding MA abuse during pregnancy and food

consumption, early studies in adult rats showed that tolerance to MA can be slow to develop (Kandel *et al.*, 1975). In contrast to this we found that these effects were transient and only observed at the start of the MA dosing period. The appetite suppressant effects of MA are no longer observed by the last week of the dosing period when both body weight and food consumption are comparable to the control dams for all MA treatment groups. Food and water consumption also decreased significantly in all groups during the last few days preceding birth. This pattern of food and water intake has been previously observed (Benson and Morris, 1971) and seems to be a normal phenomenon. It has been previously shown that maternal behaviour can be impaired by MA treatment during pregnancy (Slamberova *et al.*, 2005a, Slamberova *et al.*, 2005b). Behaviours affected by such exposure include; time spent in the nest, time spent in contact with the pups and time spent grooming the pups. These altered behavioural patterns can therefore have a significant impact on offspring development. Although maternal behaviour was not analysed in the present study we have previously observed in our lab that chronic MA given orally at a dose of 3.75 mg/kg does not impair maternal behaviours mentioned above, in the observation or retrieval test (unpublished data). Therefore, any effects found on the offspring are most likely due to the drug treatment alone and not due to any altered maternal behaviour.

Clinical studies report increased cases of premature births due to MA abuse (Nguyen *et al.*, 2010), but our results show that all groups had a similar gestation length which was approximately 22 days and this lack of effect of MA on gestation length has been found in other preclinical studies (Hruba *et al.*, 2009b, Slamberova *et al.*, 2007).

An important finding in this study was the cases of neonatal death during the postnatal period. This was unexpected as the aforementioned dose of MA was relatively low, being equivalent to 45 mg when extrapolated to humans (Reagan-Shaw *et al.*, 2008). The numbers of stillborn pups was unaffected by MA treatment in the present study yet previous work by Acuff-Smith *et al.* (1996) showed that early drug administration during pregnancy (GD 7-12) resulted in an increase of stillborn pups, albeit at a higher dose of MA (20 mg/kg) and via the subcutaneous route. The incidence of pups eaten in the postnatal period by the postnatal MA group and the prenatal/postnatal MA group exceeded that of the controls and what is considered a 'typical' amount of neonatal death with over 15% and 30% of the pups eaten respectively. As mentioned previously, our lab has shown that this dosing regime and dose of MA does not impair maternal behaviours (unpublished data) and so these effects are not thought to be caused by an impairment in the mother's behaviour. There are no studies showing the effect of oral MA on neonatal death. Therefore, our only comparison is with the s.c. MA administration. Other studies employing a dose of 5 mg/kg (sc) throughout pregnancy (Slamberova *et al.*, 2006, Chen *et al.*, 2010) or from GD 7 to 20 (Cho *et al.*, 1991) stated that mortalities of the offspring were not altered by drug treatment. At higher doses, (20-40 mg/kg via sc) postnatal mortality has been reported with a greater incidence after late drug administration in pregnancy, i.e. GD 13-18 (Acuff-Smith *et al.*, 1996) however this may be expected at such high doses of MA. To our knowledge, there are no other studies that have reported a prevalence of offspring mortality at lower doses of MA (i.e. <10 mg/kg). There are also, to the best of our knowledge, no other studies investigating MA exposure during lactation alone and so there is no comparison for our results. Nonetheless, it is a cause for concern that large numbers of offspring are

being consumed in the postnatal period after exposure to such a low dose of MA and so future work should aim to investigate these lower doses.

The somatic development of the pups highlighted some developmental delays after exposure to MA. The results of the pinna unfolding highlighted the effects of the MA treatment. On PND 4, all MA groups had less pups with pinna unfolded regardless of sex compared to their control, although this was more dramatic in the prenatal/postnatal MA group. While a delay of one day preclinically (in a rat) may seem like a short time one must consider how this may translate clinically and how it may affect the growing infant. Similar delays in somatic development have previously been reported (albeit at a higher dose of 5 mg/kg sc throughout gestation and lactation) for example in eye opening (Pometlova *et al.*, 2009, Hruba *et al.*, 2009b). Birth weights were similar across treatment groups, and stunted growth was not apparent in later life for any of the MA treated groups with body weight and body weight gain showing similar across treatments. A growth difference has been previously documented after prenatal MA treatment by Cho *et al.* (1991) where at a dose of 3 and 4.5 mg/kg male pups gained less weight compared to controls or by Hruba *et al.* (2009b) where at a dose of 5 mg/kg prenatally male and female pups both gained less weight but again there is no comparison for postnatal exposure. These delays in somatic development were also apparent in fur appearance and even in later life on PND 14 for eye opening. Fur appearance and eye opening were impeded for all MA treatment groups with eye opening being the most severely delayed regardless of the time of MA exposure. When comparing exposure times and the delays observed in somatic development, it is quite evident that the combined treatment (MA/MA) has the most severe outcome. However, between

prenatal and postnatal MA treatments it appears that the prenatal MA treatment has a greater consequence regarding somatic development.

The behavioural results also emphasized the effects of the prenatal MA and/or postnatal MA treatments on the rat offspring. Considering firstly the prenatal MA group, there was a significant behavioural impairment in the surface righting for male pups with only half being able to perform the test on PND 3. This is in comparison to the control male pups, which could all perform the test on this day. Moving further into the postnatal period, the prenatal MA group did not exhibit these behavioural impairments in the inclined plane test. Interestingly the female prenatal MA group showed similar impairments in the surface righting test but later in the inclined plane test they actually performed better compared to the controls. This phenomenon is repeated in the forelimb grip when the male prenatal MA group performs better than controls on PND 17 and the female prenatal MA group performs better than controls on PND 14. These results illustrate that regardless of sex, prenatal MA exposure has deleterious effects on neonatal behaviour but as this is occurring on different postnatal days, it appears that there may be a sex difference in the neurotoxicity of MA. This is in line with other studies that have shown even in adulthood prenatal MA exposure has different consequences for males and female offspring (Slamberova *et al.*, 2014).

Considering the postnatal MA group, there was also significant behavioural impairments but this was more apparent in the later postnatal period. In the surface righting for males less MA pups could perform the test on PND 3 in comparison to controls. The postnatal MA group however did not display these behavioural impairments in the inclined plane test. Interestingly, the female pups on PND 9 and

the male pups on PND 11 from the MA postnatal groups actually performed better on their respective days with more pups being able to perform this task compared to controls. Similar to the prenatal treatment groups in the forelimb grip the male and female postnatal MA groups showed significant impairments compared to controls on PND 14. Then on PND 17, the male postnatal MA group perform better than controls. However, the improved performance in the postnatal MA group may be more related to the poor performance by the control group rather than to the MA exposure. While prenatal and postnatal MA both have a comparable profile of behavioural impairments, the impairments seem to be more pronounced in the postnatal MA treatment group. Additionally, the time periods in which these occur differ slightly. The prenatal MA effects seem to be more noticeable in the early postnatal period (PND 2-9) whereas the postnatal MA effects seem to be more obvious in the later postnatal period (PND 7-14).

When these treatments are combined and MA is administered throughout the prenatal and postnatal period then the impairments and delays in development and behaviour are much more exaggerated as would be expected. Even with only a small number of litters remaining to represent this treatment group the surface righting showed that for each day of testing this group performed worst and not all pups could complete the task by the end of the testing period. This was seen regardless of sex. Both males and females had a similar performance to the control groups on PND 9 in the inclined plane test however, when re-exposed to the test on PND 11 their performance was unchanged and had not improved whereas all other groups were performing the test better on PND 11.

In conclusion, this study demonstrates that regardless of the period of abuse (i.e. prenatal or postnatal) these exposure periods have a negative impact on the unborn and new-born offspring in both somatic and behavioural development. The prenatal MA treatment appears to have a greater consequence on somatic development of the offspring while behavioural development of the pups is more affected by postnatal MA treatment. This may be due to the different developmental events that occur during each of the exposure times. In rodents, development of the axial skeleton, limbs, eyes and ears typically occurs *in utero* (Daston *et al.*, 2004) and this may explain why these parameters are more susceptible to alteration by the prenatal MA treatment. On the other hand, it is known that development of the cerebellum, which coordinates posture, balance and coordination, occurs postnatally in rodents (Daston *et al.*, 2004) and so is more susceptible to alteration by the postnatal MA treatment.

When we consider the clinical scenario, it suggests that if a mother was to remain abstinent from MA during pregnancy her child may still suffer developmentally if MA is abused during breastfeeding. The developmental delays are comparable to the delays seen after prenatal MA. However if a mother continues MA use throughout pregnancy and breastfeeding then these retardations are even more extreme for the offspring. Future work should aim to assess the risks associated with other exposure patterns during pregnancy and breastfeeding to give us more information on how the severity of neonatal outcome relates to the frequency and quantity of MA exposure.

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Chapter 4:

Acute and intermittent methamphetamine exposures during pregnancy have no significant impact on neonatal development and behaviour in rat offspring

Abstract

Many patterns of MA use have been identified among pregnant MA-using females; correspondingly, such patterns of use may lead to different outcomes for the neonate. The severity of effects on the neonate may be related to the frequency and quantity of MA that the foetus is exposed to. There is a paucity of studies investigating different patterns of exposure for pregnant females taking MA, either clinically or in laboratory animals. Thus, the aim of this study was to determine if acute or intermittent MA exposure during pregnancy at a pharmacological dose affects neurodevelopment and behaviour in the rat offspring during the neonatal period. Pregnant Sprague-Dawley dams (n=9-12 dams/group) received MA (3.75 mg/kg) or control (distilled water) via oral gavage on a single gestation day (21) or on multiple gestation days (6&7, 13&14 and 20&21). A range of well-recognised neurodevelopmental parameters were examined in the offspring at neonatal period. A significant increase in neonatal mortality was observed in both MA exposure patterns but no substantial impairments were noted in neurodevelopmental or behavioural parameters. This study demonstrates that MA, when given at a dose of 3.75 mg/kg on these specified time points (acutely on gestation day 21 or intermittently on gestation days 6&7, 13&14 and 20&21), has no profound effect on neonatal outcome.

4.1 Introduction

There are many patterns of MA use that exist in pregnant females such as decreased use of MA as pregnancy progresses, increased use of MA as pregnancy progresses, use of MA in one trimester only, intermittent use of MA throughout pregnancy, amongst others (Della Grotta *et al.*, 2010, Smith *et al.*, 2008, LaGasse *et al.*, 2012). Different patterns of MA use during pregnancy may lead to different outcomes for the neonate. It is conceivable that the severity of the effects on neonates relates primarily to the frequency and quantity of MA that the foetus is exposed to. However, Hrebickova *et al.* (2014) discovered that the precise timepoint of MA exposure during development plays an important role. The pattern of effects in the foetus may differ depending on the timing of MA exposure and exposure during the second and third trimesters are shown to have a greater effect than in the first trimester. Clinical data shows that most pregnant females try to remain abstinent from MA throughout the first and second trimesters but relapse in the third trimester (Della Grotta *et al.*, 2010). The use of MA once or twice throughout pregnancy also occurs most frequently in the later stages of the pregnancy (Della Grotta *et al.*, 2010).

In recent years, there has been an increase in the number of preclinical studies investigating prenatal MA exposure; however, as mentioned above, most preclinical studies have only looked at repeated daily administration of MA dosing during an extended period of pregnancy, comparing MA exposure to non-MA exposure. Assessing the risks associated with other exposure patterns may provide insight into how the severity of neonatal outcome relates to the frequency and quantity of MA exposure.

Therefore, the aim of this study was to determine if MA exposure given acutely or intermittently during pregnancy at a pharmacological dose affects neurodevelopment and behaviour in the rat offspring. The hypothesis is that both acute and intermittent MA exposure at a pharmacological dose when given orally will have an adverse effect on the rat offspring.

4.2 Materials and Methods

4.2.1 Animal Housing

Adult male (300–325 g, approx. 4 months old) and female (250–300 g, approx. 4 months old) Sprague-Dawley rats were used for this study. All females were bred in-house; all males came from Charles River (Kent, U.K.) and animals were habituated for one week from arrival. After mating, all female rats were housed singly in plastic bottom cages with appropriate bedding material (Pellets, 3Rs™, UK) and additional nesting materials (unbleached cotton and Nesteldown bedding, Petworld, Galway). All animals were maintained under standard laboratory conditions under artificial 12-h light/dark cycle (lights on from 08:00h) and temperature was maintained at 20-24^{o}C with relative humidity at 35-60%. Food and water were provided *ad libitum*. Following littering, the rat pups remained with their biological dams until PND 21, at which point the pups were weaned. Cross-fostering was not employed in this study in order to mimic clinical scenarios but also to ensure that active drugs present in the MA mother are not passed onto the control pups via breastmilk or urinary and faecal excretions (McDonnell-Dowling and Kelly, 2015b). All experiments were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway (12/NOV/07) and in compliance with the European Communities Council directive 86/609.

4.2.2 Mating

For this study, 40 female rats and 12 male rats were acquired. A male:female ratio of 1:3 for mating was used. Each cage of female rats (3) were housed overnight with one sexually mature male rat. At the beginning of the light phase on the following morning, vaginal smears were obtained from all females to check for the presence of sperm. All smears were examined under a light microscope. GD 0 was deemed the day that sperm was present in the smear. Of the 40 females that were mated with males, 33 females became pregnant (83% success rate).

4.2.3 Gestation Period and Deliveries

The expected day of delivery (birth) in rats is GD 21-22. Pregnant females were checked daily; offspring in each litter were checked and counted daily in the week after delivery to monitor for pup mortalities. The pups were randomly culled (using a random number generator) to 10 per litter on PND 1 with a litter ratio of 50:50 males:females whenever possible. One male and one female were selected for testing from each litter in order to avoid litter effects and these same pups continued through all neonatal testing stages. Pups were selected that best represented the average weight of the litter. Each of the selected pups were injected intradermally with black India ink in the footpad for unique identification purposes in a litter.

4.2.4 Drug Treatment

Methamphetamine HCl was purchased from Sigma Aldrich (Wicklow, Ireland; M8750). Rats were assigned randomly to control or MA-treated groups based on body weights and likelihood of pregnancy. The dose of MA given was 3.75 mg/kg at a volume of 1 ml/kg and controls received the VEH alone, i.e. 1 ml/kg distilled

water. Our previous dose response study (McDonnell-Dowling *et al.*, 2014) aided in deciding this dose as it compares to a clinically relevant dose in the human scenario and the use of an allometric scale takes into account the body weight and body surface area of a pregnant human versus a pregnant rat (Reagan-Shaw *et al.*, 2008) therefore maintaining the viability of the project and its comparison to clinical situations. For MA or control treatments, the dams were dosed via oral gavage once daily at 14.00h from GD 7 until PND 21 (time of weaning). Oral gavage was used as this represents the most common route of MA administration in humans (U.S. Department of Health and Human Services, 2006), but has been disregarded in preclinical investigations. Three treatment groups were determined: during the gestation and postnatal periods one group of mothers received VEH (control); the next group of mothers was given a single dose of MA (acute) on GD 21 and on all other days was given VEH; the intermittent group of mothers was given MA twice in each gestational week on GD 6 and 7, 13 and 14, 20 and 21 and on all other days was given VEH. The time point of the acute dosing on GD 21 was chosen as MA use once or twice in pregnant females most frequently occurs in the late stages of the pregnancy (Della Grotta *et al.*, 2010). Both MA treatment groups therefore received their last MA injection on the same GD.

4.2.5 Maternal Measurements

Maternal body weight was recorded daily from GD 0 to PND 21 prior to dosing of each rat (between 14.00 and 16.00h). Maternal food and water consumption were also recorded daily from GD 0. On PND 21 (day of weaning), all dams were sacrificed by decapitation.

4.2.6 Maternal Behaviour

Observational Test

Maternal behaviour was observed on PND 10 for 50 min in the home cage. The time of observation was at the beginning of the light phase between 0800-0900 h. This method was adapted from previous studies by Slamberova *et al.* (Slamberova *et al.*, 2007, Slamberova *et al.*, 2005a, Slamberova *et al.*, 2005b). During the observation test, the mother and litter were observed for 5 sec at 5-min intervals (a total of 10 observations) and the activities of the mother was recorded. During the test, a score of 1 was given if a behaviour occurred and a score of 0 was given if it did not. A total of 15 behaviours were scored including if the mother was: arched nursing, blanket nursing, passive nursing, in the nest, out of the nest, in contact with the pups, grooming the pups, carrying the pups, moving the nest or bedding, resting with eyes closed, eating, drinking, grooming, sniffing and rearing.

Retrieval Test

The mothers were tested in the retrieval test immediately after observation test on PND 10 between 09:00-10:00 h. This method was also adapted from previous studies by Slamberova *et al.* (Slamberova *et al.*, 2007, Slamberova *et al.*, 2005a, Slamberova *et al.*, 2005b). The pups were removed from their home cage and placed into a new clean cage on a heating pad for 5 min. The litter was then returned and dispersed around the home cage. During the retrieval test, the mother and litter were observed for 10 min and the activities of the mother were recorded. During the test, a score of 1 was given if a behaviour occurred and a score of 0 was given if it did not. A total of 7 behaviours were scored including: latency to carry the first pup, latency to return the first pup to the nest, latency to return all the pups to the nest, removing a

pup from the nest that was previously returned, intensive caring for the pups before placing them in the nest and extensive disruption of the nest or bedding.

4.2.7 Development of Offspring

The development of the offspring involved examining somatic development and behavioural testing. The day on which each test was performed relates to the time at which this development milestone normally occurs in rats and each test has a specific PND. Both dam and pups remained in the home cage room while testing occurred. At the time of testing, the dams were removed from the home cage and placed in a separate cage. The pups were taken directly from the home cage and placed back into the home cage after testing was completed.

Somatic Development

Somatic parameters included pinna (ear) unfolding, fur apperance, eye opening, ano-genital distance, body lengths and body weights. Pinna unfolding was recorded from PND 3, eye opening was recorded from PND 14 and fur appearance was recorded from PND 3 for males and females. The time of first appearance of fur was considered the first day of occurrence, whilst in the cases of pinna unfolding and eye opening, both pinna or eyes must unfold or open respectively to denote the first day of appearance. Recording of these parameters continued until all pinna had unfolded, eyes had opened and fur was present in all rat pups.

Ano-Genital Distance

Ano-genital distance was measured (for comparison) for possible masculinising or feminising effects of MA. This was measured using a digital calipers between the base of the genitals and the top of the anus. This was performed on PND 3 and 24.

Body Length

Body length was measured for each pup to compare growth sizes. This was measured using a digital calipers between the tip of the nose and the base of the tail. This was performed on PND 7 and 14.

Body Weight

Body weight was measured for each pup throughout the neonatal period on PND 1, 2, 4, 8, 11, 15, 18 and 21 prior to behavioural testing each day.

Behavioural Testing

Righting Reflex on a Surface

Pups were placed in the supine position on a flat surface and the time taken to turn over and restore its normal prone position (on all fours) was recorded. The maximum time allowed was 30 s. A time of 30 s was recorded if the pup did not right itself within this period and the test was terminated. This test was performed on PND 2, 3, 4 and 5.

Inclined Plane

The inclined plane apparatus consists of a flat timber surface that is inclined and held at a 30˚ angle. The pups were placed facing downward at the top of the inclined plane apparatus. The time taken to turn 180˚ and face upward was recorded. The maximum time allowed was 30 s. A time of 30 s was recorded if the pup did not turn within this period and the test was terminated. This test was performed on PND 9 and 11.

Forelimb Grip

The forelimb grip apparatus consists of a thin steel bar supported by two adjustable poles. The bar is approx. 20 cm in length, 0.2 cm in diameter and lies 25 cm above

the base of the platform. The pup was gripped at the base of the tail and lowered onto the bar. The length of time the pup was able to hold on to the bar before falling was recorded and the maximum time allowed was 30 s. A time of 30 s was given if the pup did not fall within this period and the test was terminated. This test was performed on PND 14 and 17.

4.2.8 Statistical Analysis

All figures representing the data from the testing period were constructed using Graphpad Prism 5.0. The data were then analysed using the statistical package SPSS 20.0. Data were firstly assessed to determine if it displayed normality of distribution and homogeneity of variance (Shapiro–Wilks and Levene's test $p>0.05$). The data were also assessed to determine if it displayed sphericity (Mauchly's test $p>0.05$) however, if this failed then the Greenhouse–Geisser correction was used (i.e. degrees of freedom corrected). For the parametric data, tests used included: repeated measures ANOVA to compare the overall effect for related data, Two-Way ANOVA to compare the effect of treatment groups and sex, One-Way ANOVA and Student-Newman Keuls *post-hoc* tests were used to define where the significance lay. For the non-parametric data tests used included: Friedman's ANOVA by Ranks to compare the overall effect for related data, Wilcoxon Match-Pairs test to compare the effect of time, Kruskal-Wallis test to compare the effect of treatment groups and Mann-Whitney U tests were used to define where the significance lay. For pinna unfolding, eye opening and fur appearance the data were taken as counts (present or absent) and for surface righting, inclined plane test and forelimb grip the data were taken as counts (ability to perform the test or not) and so the Chi-Squared test was performed for these parameters. All results reported are for the MA treatment groups compared

to the control group. The level of significance was set at $p<0.05$ for all parameters except for the Chi-Squared test which was set at $p<0.01$ (due to multiple comparisons).

4.3 Results

4.3.1 Prenatal Maternal Daily Measurements

Prenatal Maternal Weight Gain

A significant effect of gestation day was found ($F_{(2.01, 60.39)}=948.38$, $p<0.001$) with all groups gaining weight as gestation progressed. No significant effect of treatment was found. A significant interaction effect of gestation day and treatment was found ($F_{(4.03, 60.39)}=6.46$, $p<0.001$). No significant effect of treatment was found for pre-dosing body weight gain or body weight gain in the first week of dosing, but a significant effect of treatment was found for body weight gain in the second week of dosing ($F_{(2, 30)}=6.51$, $p<0.01$). A *post-hoc* test revealed that the intermittent group gained less weight in the second dosing week compared to the control group (Figure 4.1).

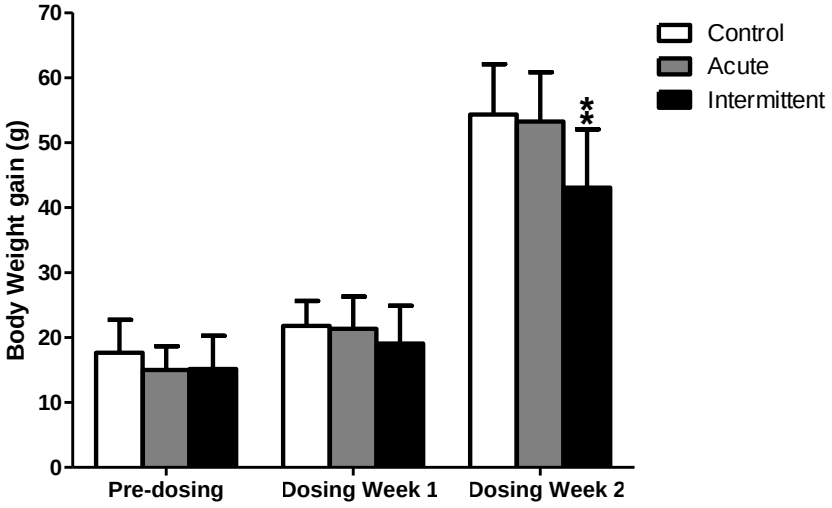

Figure 4.1: Maternal weight gain during gestation. Total weight gain during gestation from the pre-dosing and post-dosing periods (n=9-12/group). Data are expressed as Mean+SD, $^{**}p<0.01$ vs. control.

Prenatal Maternal Food Consumption

A significant effect of gestation day was found ($F_{(4.93,\ 133.20)}=63.30$, $p<0.001$) with food consumption increasing slightly throughout the gestation period and then decreasing for the last few days preceding birth (GD18 to 20). A significant interaction effect of gestation day and treatment was found ($F_{(9.87,\ 133.20)}=2.56$, $p<0.01$). No significant effect of treatment was found (Figure 4.2). No significant effect of treatment was found for total food consumption in the pre-dosing or dosing weeks (data not shown).

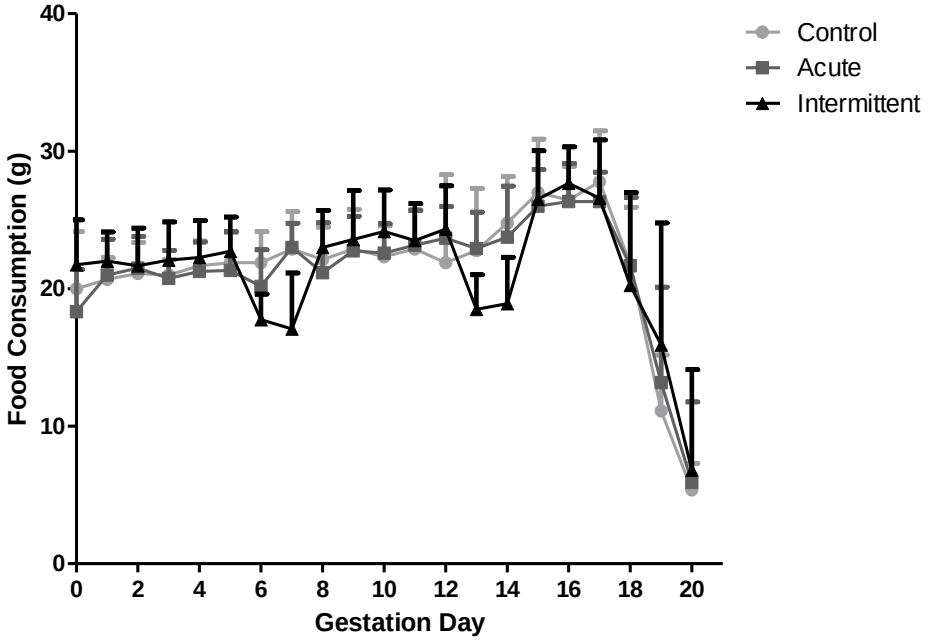

Figure 4.2: Maternal food consumption during gestation. Food consumption for each day of gestation (n=9-12/group). Data are expressed as Mean+SD.

Prenatal Maternal Water Consumption

No significant effect of gestation day, treatment or interaction effect of gestation day and treatment was found for water consumption. No significant effect of treatment was found for total water consumption in the pre-dosing or dosing weeks (data not shown).

4.3.2 Maternal and Litter Observations

No significant effect of treatment was found for day of birth/length of gestation period with all groups having a gestation length of 22 days. No significant effect of treatment was found for number of males or females in a litter (data not shown). A significant effect of treatment was found for number of pups in a litter ($K=6.85$, $p<0.05$) with the intermittent group having less pups in a litter compared to the

control group (Table 4.1). Maternal death did not occur in any treatment groups. No significant effect of treatment was found for percentage of stillborn pups, percentage of pups that died in postnatal period or total percentage of pup deaths in the postnatal period. A significant effect of treatment was found for percentage of pups eaten in the postnatal period (K=10.81, $p<0.01$) in that the acute and intermittent groups had a higher percentage of pups eaten compared to the control group (Table 4.1). Based on the high percentage of pup mortalities in the MA groups the final number of litters used for testing was six control litters, five acute litters and six intermittent litters. The final number of litters used for testing was therefore 17.

Group	No. of Pups/Litter	% Pups Stillborn	% Pups Found Dead	% Pups Eaten	% Total Deaths
Control	15 ± 2	8.5	13.0	0.0	21.5
Acute	14 ± 2	9.3	30.2	22.2**	61.7
Intermittent	12 ± 4*	6.4	14.5	25.1**	46.0

Table 4.1: Litter observations. Litter observations at birth and in the postnatal period (n=9-12 dams/group). Data are expressed as Mean±SD or mean % of pups; **$p<0.01$, *$p<0.05$ vs. control.

4.3.3 Postnatal Maternal Daily Measurements

Postnatal Maternal Weight Gain

A significant effect of postnatal day was found ($F_{(3.66, 32.92)}=48.61$, $p<0.001$) with all groups gaining weight as time progressed. No significant effect of treatment or interaction effect of postnatal day and treatment was found. No significant effect of

treatment was found for body weight gain in the first or second postnatal week but a significant effect of treatment was found for body weight gain in the third postnatal week ($F_{(2, 14)}$=3.77, p<0.05). A *post-hoc* test showed that the intermittent group gained less weight in the third postnatal week of dosing compared to the control group (Figure 4.3).

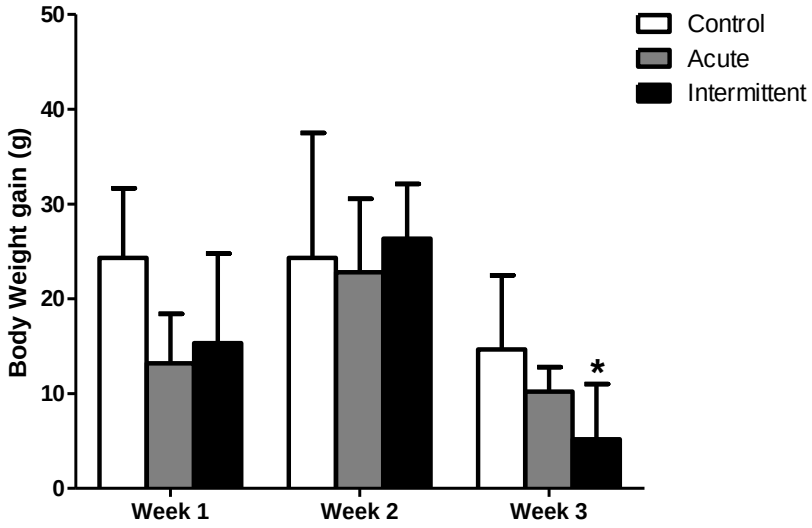

Figure 4.3: Maternal weight gain during lactation. Total weight gain from postnatal weeks 1, 2 and 3 (n=5-6/group). Data are expressed as Mean+SD, *p<0.05 vs. control.

Postnatal Maternal Food Consumption

A significant effect of postnatal day was found ($F_{(5.11, 51.09)}$=136.23, p<0.001) with food consumption increasing for all groups as time progressed. No significant effect of treatment or interaction effect of postnatal day and treatment was found (Figure 4.4). No significant effect of treatment was found for total food consumption in the first, second or third postnatal week (data not shown).

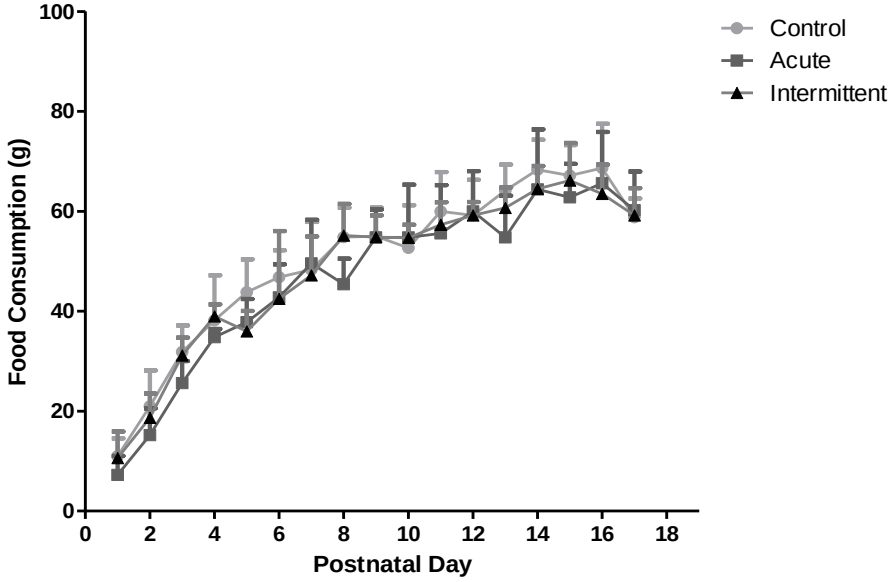

Figure 4.4: Maternal food consumption during lactation. Food consumption for each day of lactation (n=5-6/group). Data are expressed as Mean±SD.

Postnatal Maternal Water Consumption

A significant effect of postnatal day was found ($F_{(1.51,\ 4.52)}$=15.57, p<0.05). No significant effect of treatment or interaction effect of postnatal day and treatment was found. No significant effect of treatment was found for total water consumption in the first, second or third postnatal weeks (data not shown).

4.3.4 Maternal Behaviour

A significant effect of treatment was found for rearing in the observational test ($F_{(2,\ 14)}$=3.97, p<0.05). A *post-hoc* test showed that the intermittent group reared more times compared to the control group (data not shown). No significant effect of treatment was found for the other maternal behaviours in the observational test (time spent nursing (passive and active), time in and out of nest, time in contact, grooming

and carrying pups, moving of bedding/nest, resting, eating, drinking, grooming and sniffing).

A significant effect of treatment was found for time taken to place the first pup back into the nest in the retrieval test ($K=6.08$, $p<0.05$). A *post-hoc* test showed that the intermittent group took less time to return the first pup to the nest compared to the control group (data not shown). No significant effect of treatment was found for the other maternal behaviours in the retrieval test (time to carry first pup and return all pups to nest, removing of pup from the nest, intensive care for the pups, moving bedding/nest) .

4.3.5 Somatic Development of Offspring

Birth Weights

No significant effect of sex, treatment or interaction effect of treatment and sex was found (data not shown).

Pinna Unfolding

No significant effect of sex or treatment was found on PND 3 or 4 (Table 4.2).

Drug	PND 3 (%)	PND 4 (%)
Male		
Control	0	83
Acute	20	100
Intermittent	33	83
Female		
Control	0	83
Acute	0	100
Intermittent	17	67

Table 4.2: Pinna unfolding. Percentage of pups with pinna unfolded for male and female pups (n=5-6/group). Data are expressed as percentage of pups with both pinna unfolded.

Fur Appearance

No significant effect of sex or treatment was found on PND 3 or 4 (data not shown).

Eye Opening

No significant effect of sex or treatment was found on PND 14, 15 or 16 (Table 4.3).

Drug	PND 14 (%)	PND 15 (%)	PND 16 (%)
Male			
Control	0	17	100
Acute	20	60	60
Intermittent	0	67	100
Female			
Control	0	33	100
Acute	20	80	80
Intermittent	0	67	83

Table 4.3: Eye opening. Percentage of pups with eyes open for male and female pups (n=5-6/group). Data are expressed as percentage of pups with eyes open.

Ano-Genital Distance

A significant effect of sex was found on PND 3 ($F_{(1, 28)}$=118.75, p<0.001) and PND 24 ($F_{(1, 28)}$=76.20, p<0.001) with females having a smaller ano-genital distance compared to males. No significant effect of treatment or interaction effect of treatment and sex was found on either PND (data not shown).

Body Length

No significant effect of treatment, sex or interaction effect of treatment and sex was found on any of the testing days (data not shown).

Neonatal Body Weight

A significant effect of postnatal day was found ($F_{(1.99, 55.58)}$=610.73, p<0.001) with all groups gaining weight as time progressed. A significant interaction effect of postnatal day and treatment was found ($F_{(9.93, 55.58)}$=2.50, p<0.05). No significant effect of treatment was found. No significant effect of sex or interaction effect of

treatment and sex was found for body weight gain in the postnatal period (PND 1-21) but a significant effect of treatment was found ($F_{(2, 28)}$=586.91, p<0.01). A *post-hoc* test showed that there was no difference between treatment groups compared to control for body weight gain (data not shown).

4.3.6 Behavioural Testing of Offspring

Surface Righting

A significant effect of sex was found on PND 5 (X_1^2=4.00, p<0.05) with females performing better than males. A significant effect of treatment was found on PND 5 (X_1^2=11.31, p<0.05) (Table 4.4). A *post-hoc* test showed that females in the acute group had less pups that could reach the criterion of 10 s compared to the control group (p<0.01) on PND 5.

Drug	PND 2 (%)	PND 3 (%)	PND 4 (%)	PND 5 (%)
Male				
Control	67	50	100	50
Acute	40	40	60	100
Intermittent	33	33	100	67
Female				
Control	83	33	50	100
Acute	20	60	40	20**
Intermittent	33	17	50	67

Table 4.4: Surface righting. Surface righting for male and female pups on PND 2, 3, 4 and 5 (n=5-6/group). Data are expressed as percentage of pups that complete the test in less than 10 s; **p<0.01 vs. relevant control.

Inclined Plane

No significant effect of sex or treatment was found on PND 9 or 11 (Figure 4.5).

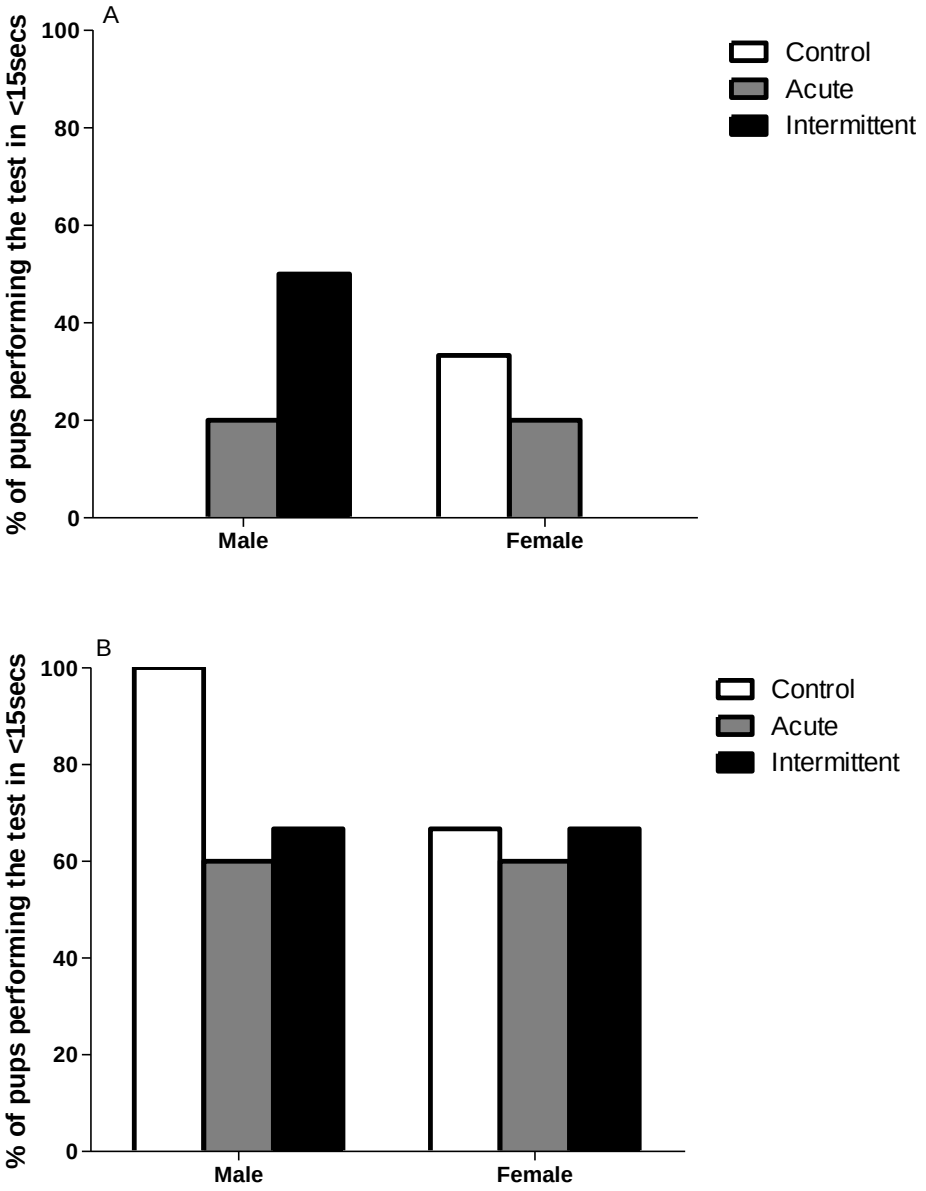

Figure 4.5: Inclined plane. Inclined plane for male and female pups on (A) PND 9 and (B) PND 11 (n=5-6/group). Data are expressed as percentage of pups that complete the test in less than 15 s.

Forelimb Grip

No significant effect of sex or treatment was found on PND 14 or 17 (Figure 4.6).

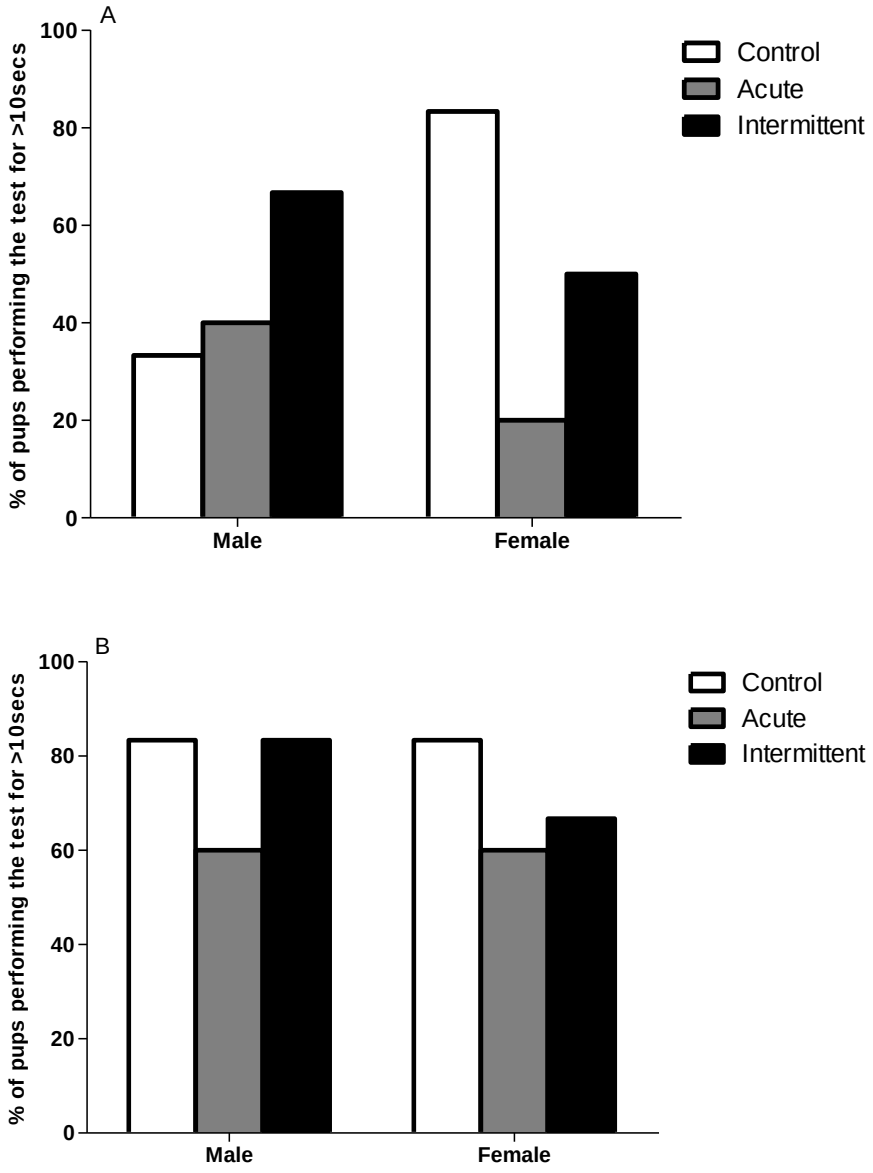

Figure 4.6: Forelimb grip. Forelimb grip for male and female pups on (A) PND 14 and (B) PND 17 (n=5-6/group). Data are expressed as percentage of pups that could perform the task for more than 10 s.

4.4 Discussion

The aim of this study was to determine if MA exposure given acutely or intermittently during pregnancy at a pharmacological dose affects neurodevelopment and behaviour in the rat offspring. The results illustrate that intermittent and acute *in utero* exposure of MA does not trigger any neurodevelopmental or behavioural deficits during the neonatal period.

The first objective of this study was to determine if prenatal MA exposure given acutely or intermittently has any consequences on maternal parameters. Prenatal body weight gain was altered by intermittent MA exposure in that mothers gained less weight in the third gestation week compared to the control mothers. This effect was also observed in the postnatal period where the same mothers gained less weight in the third postnatal week preceding weaning. Although no other studies have investigated these dosing regimens, previous studies showed that chronic prenatal MA (albeit at a higher dose of 5 mg/kg) has no consequence on maternal weight gain (Slamberova *et al.*, 2005a, Malinova-Sevcikova *et al.*, 2014) but higher doses of MA (>10 mg/kg) resulted in significantly lower average weights for the mothers during gestation (White *et al.*, 2009). While it might be postulated that the reduction in body weight gain observed in the present study may be due to reduced food intake, the results show that for both MA groups, food consumption and water consumption remained unaltered during the prenatal and postnatal periods. The correlation may therefore be owed to increased locomotor activity but unfortunately this was not examined in the present study and may warrant further investigation.

Looking next at the litter characteristics at birth and in the postnatal period, the most evident outcome of MA exposure was seen in the pup mortalities. For both acute and

intermittent treatment groups the percentages of pups eaten surpassed that of the control group by over 20% in both cases. This phenomenon contradicts our previous study that showed no difference in pup mortalities after chronic prenatal MA at a dose of 2.5 mg/kg but this is more than likely due to the lower dose of MA (McDonnell-Dowling *et al.*, 2014). With higher MA doses and different routes of administration this outcome has also been reported (Vorhees *et al.*, 2005, White *et al.*, 2009). It was of great concern that administration of this low dose of MA to the mothers caused them to consume their offspring, and suggested that the effects on the pups may be maternally mediated rather than drug mediated. Although cross-fostering has been previously shown to attenuate effects of prenatal MA exposure (Pometlova *et al.*, 2009) this was not employed due to dosing in the late gestation period. MA clearance is decreased in late gestation and the half-life of the drug is increased (White *et al.*, 2011) which would suggest that pups may receive some amount of MA *via* the breast milk, urinary or faecal excretion (McDonnell-Dowling and Kelly, 2015). Thus, maternal behaviour was investigated in the postnatal period. Traditionally, the observation and the retrieval tests are performed daily from birth until PND 12. For the present study, a 'snapshot' of maternal behaviour was taken and these tests were performed only on PND 10. Nonetheless, the mothers exhibited similar behaviour towards their offspring in the observational test including time spent in nest, time with pups, nursing of pups, grooming of pups and other behaviours such as nest building and self-care. Again, in the retrieval test the mothers displayed no negative behaviour towards their young. As neonatal death due to MA exposure was something that occurred throughout the postnatal period then it is likely that if this was maternally mediated then this would be observed in the aforementioned tests. It is not uncommon for a rat mother to consume a pup if she

senses its imminent death and it is therefore hypothesised that the pups were severely affected by the MA exposure and so their death was forthcoming. Overall, the maternal data suggests that MA has no substantial influence on the mothers during gestation and lactation and suggests that any outcomes observed in the offspring are due to prenatal MA exposure rather than maternal alterations.

The second objective of this study was to determine if single or periodic prenatal MA exposures have an effect on somatic and behavioural development in the offspring. The prenatal MA exposures did not have a negative impact on the somatic development of the offspring and birth weights, fur appearance, ano-genital distance, body length and body weight gain were all comparable to the control group for both males and females. This has been illustrated previously at lower doses of MA (0.625 and 1.25 mg/kg po) given chronically during pregnancy (McDonnell-Dowling *et al.*, 2014) but is in contrast with higher doses of MA (5 mg/kg s.c.) given chronically during pregnancy where somatic developmental delays were evident (Slamberova *et al.*, 2006). The behavioural and reflex ontogeny results, such as surface righting, inclined plane test and forelimb grip, also indicated no overall delays in development in both males and females. Surface righting was not significantly influenced by MA exposure in that both MA treatment groups showed no major impairments when performing this task. The female acute MA group were negatively affected in that not all pups were able to complete this task even though they were subjected to multiple exposures of the test, although this is only noted on the last day of testing (PND 5). Deficits in postural reflexes and sensorimotor function are previously reported however in other studies (McDonnell-Dowling *et al.*, 2014, Hruba *et al.*, 2008, Slamberova *et al.*, 2006). The inclined plane test also revealed no significant

behavioural impairments in either MA exposure groups but there is a noteworthy trend on PND 11 where both male MA exposure groups seem to have less pups performing the task. This is in agreement with Cho *et al.* (1991) that found the durations required for the development of negative geotaxis was significantly longer after chronic exposure to s.c. MA at 3 and 4.5 mg/kg *in utero*. In addition to this, the forelimb grip uncovered no behavioural deficits in either the acute or intermittent MA treatments but once more, there was a trend in that the females appeared to be affected by both MA treatments on PND 14 with less pups performing the task. The forelimb grip is a measure of strength and co-ordination. Based on the findings from Gerald and Gupta (1977) and Gerald *et al.* (1979), it was noted that amphetamines caused increased muscle weakness as a result of inhibition of transmission at the neuromuscular junction. Slamberova *et al.* (2006) remarked that alterations in performance on the bar-holding test and rotarod test may therefore be due to alterations in the motor system development rather than in the sensory system. As well as this, it should be noted that development of the cerebellum (vital for balance and co-ordination) in rats does not occur until the end of the gestation period. Consequently, the timing of dosing for both the acute and intermittent treatments occurs at a crucial time in brain development. This may explain the absence of delays in somatic developmental parameters. Previous findings nonetheless are in agreement with the present study that found no effect of prenatal MA on strength in the bar-holding test (Pometlova *et al.*, 2009, Slamberova *et al.*, 2006, Hruba *et al.*, 2009b). This demonstrates that the offspring remain unaffected by prenatal MA exposure in the said behaviours.

In conclusion, this is the first study to show that a single MA insult in late pregnancy or several doses throughout the pregnancy are not as harming to offspring as chronic

daily doses throughout the pregnancy. Our results indicate the importance of the time of exposure rather than the frequency of exposure, with prolonged MA exposures leading to neurodevelopmental delays (McDonnell-Dowling and Kelly, 2015a) compared to sporadic exposures used in this study. Therefore, if a mother is successful in reducing the amount of MA exposures throughout the pregnancy, the child may not be in as much danger of displaying developmental delays in early life.

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Chapter 5:

Does route of methamphetamine exposure during pregnancy have an impact on neonatal development and behaviour in rat offspring?

McDonnell-Dowling, K. & Kelly, J. P. 2015. Does route of methamphetamine exposure during pregnancy have an impact on neonatal development and behaviour in rat offspring? *International Journal of Developmental Neuroscience*, 49, 14-22.

Abstract

Many preclinical studies have aimed to elucidate the effects of MA exposure during pregnancy on the offspring in recent years. However, the severity of effects on the neonate may be related to the sc route of administration of the drug that is often employed (88% of preclinical studies) and consequently the delivered dose that the foetus is exposed to. To date there is a paucity of comparative studies investigating different routes of administration for MA during pregnancy and it is not known how these different routes compare when it comes to neonatal outcome. Thus, the aim of this study was to determine if the route of administration of MA (oral gavage or sc injection) during pregnancy at a pharmacological dose affects the magnitude of neurodevelopmental and behavioural effects in the resultant rat offspring. Pregnant Sprague-Dawley dams (n=10 dams/group) received MA (3.75 mg/kg) or control (distilled water) via oral gavage or sc injection from gestation day 7-21. A range of well-recognised neurodevelopmental parameters were examined in the offspring. When administered sc, MA significantly reduced maternal weight gain and altered maternal behaviour; mothers spent less time in the nest with pups and spent less time nursing compared to controls. Significant impairments in neurodevelopmental parameters were evident in both MA treatment groups. Somatic development such as pinna unfolding, fur appearance and eye opening were all delayed after MA exposure but these impairments were more pronounced in the MA sc group. Other somatic parameters such as ano-genital distance and body length were only impeded by sc MA. Behavioural development in the surface righting, inclined plane and forelimb grip tests were also altered for both MA treatment groups. This study demonstrates that prenatal MA can have a profound effect on neonatal outcome, but this can be exacerbated if given via the subcutaneous route, as well as producing

additional effects not seen with the oral route. Consequently, the route of administration should be considered when interpreting preclinical studies investigating prenatal MA exposure.

5.1 Introduction

The World Health Organization has estimated that over 35 million people abuse MA globally on a regular basis, considerably more than the estimates for cocaine (15 million) or heroin (10 million) (Golub *et al.*, 2005). The rise in popularity of MA is thought to be related to a shift from amphetamine and heroin use due to supply shortages and also the greater availability of high purity crystalline MA (National Drug & Alcohol Research Centre, 2007, United Nations Office on Drugs and Crime, 2012). Approximately 50% of those who abuse drugs are female (Anderson and Choonara, 2007) and a study of pregnant female MA users and nonusers in specific geographic areas in the US known to have high MA usage showed that over 5% of females used the drug during pregnancy (Arria *et al.*, 2006). This statistic only represents the amount of females that admitted to MA use druing pregnancy and since underreporting is common then the true prevalence of MA use during pregnancy may be higher than what has been reported.

In recent years, there has been an increase in the number of preclinical studies investigating prenatal MA exposure (Bernaskova *et al.*, 2011, Bubenikova-Valesova *et al.*, 2009, Chen *et al.*, 2010, Hrebickova *et al.*, 2014, Malinova-Sevcikova *et al.*, 2014); however, most preclinical studies have only examined chronic daily administration of MA during pregnancy using sc injection as the route of administration (86% of preclinical studies use sc injection; (McDonnell-Dowling and Kelly, 2015), a route that has never been reported with MA in humans. In adult rats, it has been shown that there are significant route-dependent differences in the effects of MA on pharmacokinetic parameters and behaviour (Cunningham *et al.*, 2008). In this aforementioned study, the sc route of administration was shown to have the most profound effect on stereotyped behaviour and locomotor activity compared to other

routes (intraperitoneal and intravenous) and this is most likely due to the 100% bioavailability (versus 58% for intraperitoneal) and increased AUC of the drug.

According to the FDA guidelines for performing developmental and reproductive studies, the drug should be delivered by a route that most closely approximates the human route of administration (Collins *et al.*, 1999b, Collins *et al.*, 1999a). In pregnant females, MA is most commonly taken by either ingesting or smoking the drug. Oral gavage is therefore by far the most suitable route to employ. Assessing the risks associated with MA exposure during pregnancy and lactation using oral gavage as the route of administration has greater clinical relevance and this has provided much insight into how neonatal outcome can be affected in a clinically relevant animal model (McDonnell-Dowling *et al.*, 2014, McDonnell-Dowling and Kelly, 2015a). However, it is still unknown how these different routes of MA administration compare when it comes to neonatal outcome and if sc injections yield a greater effect due purely to the greater amount of delivered dose of the drug to the offspring.

Hence, the aim of this study was to determine if the route of administration of MA (oral gavage or subcutaneous injection) during pregnancy at a pharmacological dose determines the neurodevelopmental and behavioural outcome in the rat offspring. The hypothesis is that both oral and subcutaneous MA exposures will have an adverse effect on the rat offspring but that this may be augmented for subcutaneous exposure.

5.2 Materials and Methods

5.2.1 Animal Housing

Adult male (300–325 g, approx. 4 months old) and female (250–325 g, approx. 4 months old) Sprague-Dawley rats were used for this study. All females were bred in-house; all males came from Charles River (Kent, U.K.) and animals were habituated for one week from arrival. After mating, all female rats were housed singly in plastic bottom cages with additional nesting materials. All animals were maintained under standard laboratory conditions under artificial 12-h light/dark cycle (lights on from 08:00h) and temperature was maintained at 20-24^{o}C with relative humidity at 35-60%. Food and water were provided *ad libitum*. Following littering, the rat pups remained with their biological dams until PND 21, at which point the pups were weaned. Cross-fostering was not employed in this study in order to mimic clinical scenarios but also to ensure that active drugs present in the MA mother are not passed onto the control pups via breastmilk or urinary and faecal excretions (McDonnell-Dowling and Kelly, 2015c). All experiments were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway (12/NOV/07) and in compliance with the European Communities Council directive 86/609.

5.2.2 Mating

For this study, 59 female rats and 20 male rats were used. A male:female ratio of 1:3 for mating was used. Each cage of female rats (3) were housed overnight with one sexually mature male rat. At the beginning of the light phase the following morning, vaginal smears were obtained from all females to check for the presence of sperm. All smears were examined under a light microscope. Gestation day 0 was deemed

the day that sperm was present in the smear. Of the 59 females that were mated with males, 49 females became pregnant (i.e. 83% mating success rate).

5.2.3 Gestation Period and Deliveries

The expected day of delivery (birth) in rats is GD 21-22. Pregnant females were checked daily; offspring in each litter were checked and counted daily in the week after delivery to monitor for pup mortalities. The pups were randomly culled (using a random number generator) to 10 per litter on PND 1 with a litter ratio of 50:50 of males:females whenever possible. One male and one female were selected for testing from each litter in order to avoid litter effects and these same pups continued through all neonatal testing stages. Pups were selected based on weight, i.e. a representative that had the closest weight to the litter average for each sex at PND 1. Each of the selected pups were injected intradermally with black India ink in the footpad for unique identification purposes within the litter.

5.2.4 Drug Treatment

Methamphetamine HCl was purchased from Sigma Aldrich (St. Louis, MO; M8750). Rats were assigned randomly to control or MA-treated groups based on body weights and likelihood of pregnancy (n=13 for controls, n=18 for MA treatments). MA abusers generally use a dose starting at 20 mg with a common MA dose being 30 mg (Golub *et al.*, 2005). When extrapolating this dose back to a preclinical model, the use of an allometric scale (Reagan-Shaw *et al.*, 2008) takes into account the body weight and the body surface area of the human and the animal. Therefore, the MA dose to use in a preclinical model (rat) would be 2.5 mg/kg. Our previous dose response study (McDonnell-Dowling *et al.*, 2014) investigated this and similar

doses and for the present study the dose employed was slightly higher to correct for the pharmacokinetic differences for MA between rats and humans (Cho *et al.*, 2001) therefore maintaining the viability of the project and its comparison to clinical situations. The dose of MA that the animals received was 3.75 mg/kg at a volume of 1 ml/kg and controls received the VEH alone, i.e. 1 ml/kg distilled water. For MA or control treatments, the dams were dosed both via oral gavage and sc injection once daily at 14.00h from GD 7 until GD 21 (time of birth). Both routes of administration were employed to control for the stress associated with each of these routes. Oral gavage was used as this represents the most common route of MA administration in humans (U.S. Department of Health and Human Services, 2006), but has been overlooked in many preclinical investigations, in favour of sc injection which is by far the most common route of MA administration in such studies. Thus, the study comprised three treatment groups: during the gestation period one group of mothers received VEH (control) via gavage and sc routes; the next group of mothers was given MA via oral gavage and VEH via sc injection (MA gavage); the third group of mothers was given MA via sc injection and VEH via oral gavage (MA sc).

5.2.5 Maternal Daily Measurements

Maternal body weight was recorded daily from GD 0 until birth prior to dosing of each rat (between 14.00 and 16.00h). Maternal food and water consumption were also recorded daily from GD 0. On PND 21 (day of weaning), all dams were sacrificed by decapitation.

5.2.6 Maternal Behaviour

Observational Test

Maternal behaviour was observed on PND 10 in the home cage. The selection of this PND was based on previous literature that showed maternal behaviour after MA exposure was most significantly altered between PND 7 and 12 (Slamberova *et al.*, 2005a). The time of observation on PND 10 was at the beginning of the light phase between 08.00 and 10.00h for 50 min in the home cage. This method was adapted from previous studies by Slamberova *et al.* (Slamberova *et al.*, 2007, Slamberova *et al.*, 2005a, Slamberova *et al.*, 2005b). During the observation test, the mother and litter were observed for 5 sec at 5 min intervals (a total of 10 observations) and the activities of the mother was recorded. During the test, a score of 1 was given if a behaviour occurred and a score of 0 was given if it did not. A total of 15 behaviours were scored including if the mother was: arched or blanket nursing (active nursing), passive nursing, in the nest, out of the nest, in contact with the pups, grooming the pups, carrying the pups, moving the nest or bedding, resting with eyes closed, eating, drinking, grooming, sniffing and rearing. All tests were recorded onto a DVR and scored manually by an experimenter blind to treatment groups.

5.2.7 Development of Offspring

The development of the offspring involved examining somatic development and behavioural testing. The day on which each test was performed relates to the time at which this development milestone normally occurs in rats and each test has a specific PND. Both dam and pups remained in the home cage room while testing occurred. At the time of testing, the dams were removed from the home cage and

placed in a separate cage. The pups were taken directly from the home cage and placed back into the home cage after testing was completed.

Somatic Development

Somatic parameters included pinna (ear) unfolding, fur apperance, eye opening, ano-genital distance, body lengths and body weights. Pinna unfolding was recorded from PND 3, eye opening was recorded from PND 14 and fur appearance was recorded from PND 3 for males and females. The time of first appearance of fur was considered the first day of occurrence, whilst in the cases of pinna unfolding and eye opening, both pinna or eyes must unfold or open respectively to denote the first day of appearance. Recording of these parameters continued until all pinna had unfolded, eyes had opened and fur was present in all rat pups.

Ano-Genital Distance

Ano-genital distance was measured (for comparison) for possible masculinising or feminising effects of MA. This was measured using a digital calipers between the base of the genitals and the top of the anus. This was performed on PND 3.

Body Lengths

Body length was measured for each pup to compare growth sizes. This was measured using a digital calipers between the tip of the nose and the base of the tail. This was performed on PND 7.

Body Weight

Body weight was measured for each pup throughout the neonatal period on PND 1, 2, 4, 8, 11, 15 and 18 prior to behavioural testing each day.

Behavioural Testing

Righting Reflex on a Surface

Pups were placed in the supine position on a flat surface and the time taken to turn over and restore its normal prone position (on all fours) was recorded. The maximum time allowed was 30 s. A time of 30 s was recorded if the pup did not right itself within this period and the test was terminated. This test was performed on PND 2, 3, 4 and 5.

Inclined Plane

The inclined plane apparatus consists of a flat timber surface that is inclined and held at a 30˚ angle. The pups were placed facing downward at the top of the inclined plane apparatus. The time taken to turn 180˚ and face upward was recorded. The maximum time allowed was 30 s. A time of 30 s was recorded if the pup did not turn within this period and the test was terminated. This test was performed on PND 9 and 11.

Forelimb Grip

The forelimb grip apparatus consists of a thin steel bar supported by two adjustable poles. The bar is approx. 20 cm in length, 0.2 cm in diameter and lies 25 cm above the base of the platform. The pup was gripped at the base of the tail and lowered onto the bar. The length of time the pup was able to hold on to the bar before falling was recorded and the maximum time allowed was 30 s. A time of 30 s was given if the pup did not fall within this period and the test was terminated. This test was performed on PND 14 and 17.

5.2.8 Statistical Analysis

All figures representing the data from the testing period were constructed using Graphpad Prism 5.0. The data were then analysed using the statistical package SPSS 22.0. Data were firstly assessed to determine if it displayed normality of distribution and homogeneity of variance (Shapiro–Wilks and Levene's test $p>0.05$). This determines whether the data are parametric or non-parametric. The data were also assessed to determine if it displayed sphericity (Mauchly's test $p>0.05$) however if this failed then the Greenhouse–Geisser correction was used (i.e. degrees of freedom corrected). For the parametric data, tests used included: repeated measures ANOVA to compare the overall effect for related data, Two-Way ANOVA to compare the effect of treatment groups and sex, One-Way ANOVA and Student-Newman Keuls *post-hoc* tests were used to define where the significance lay. For the non-parametric data tests used included: Friedman's ANOVA by Ranks to compare the overall effect for related data, Wilcoxon Match-Pairs test to compare the effect of time, Kruskal-Wallis test to compare the effect of treatment groups and Mann-Whitney U tests were used to define where the significance lay. For pinna unfolding, eye opening and fur appearance the data were taken as counts (present or absent) and for surface righting, inclined plane test and forelimb grip the data were taken as counts (ability to perform the test or not) and so the Chi-Squared test was performed for these parameters. All results reported are for the MA treatment groups compared to the control group. The level of significance was set at $p<0.05$ for all parameters except for the Chi-Squared test which was set at $p<0.008$ (due to multiple comparison corrections).

5.3. Results

5.3.1 Prenatal Maternal Daily Measurements

Prenatal Maternal Weight Gain

A significant effect of gestation day was found ($F_{(1.89, 50.91)}$=717.89, p<0.001) with all groups gaining weight as gestation progressed. A significant interaction effect of gestation day and treatment was found ($F_{(3.77, 50.91)}$=2.75, p<0.05). No significant effect of treatment was found (data not shown). A significant effect of treatment was found for total body weight gain in the second week of gestation ($F_{(2, 27)}$=5.20, p<0.01). A *post-hoc* test revealed that both MA treatment groups gained less weight in the second week of gestation compared to the control group but this was most notable in the MA sc group (Figure 5.1).

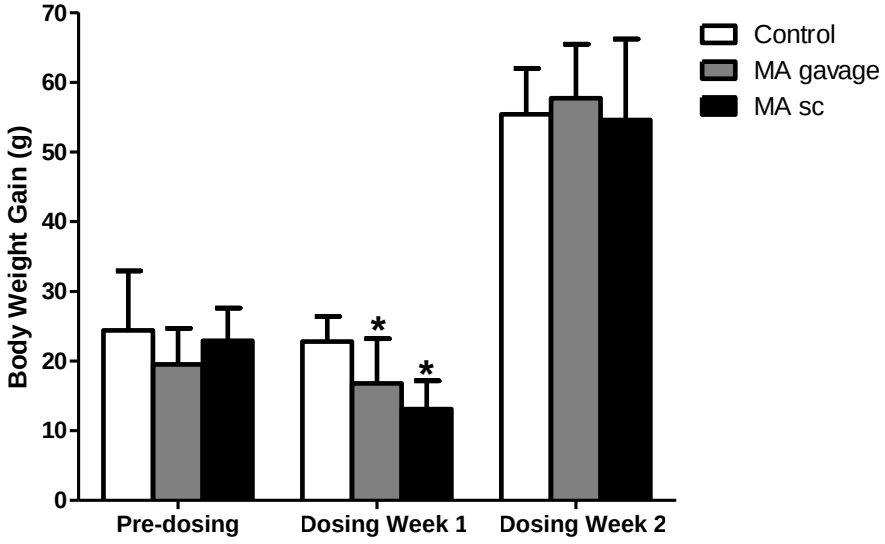

Figure 5.1: Maternal weight gain during gestation. Total weight gain from the pre-dosing and dosing periods (n=10/group). Data are expressed as Mean+SD, *p<0.05 vs. control.

Prenatal Maternal Food Consumption

No significant effect of gestation day, treatment or interaction effect of gestation day and treatment was found for daily food consumption (data not shown). A significant effect of treatment was found for total food consumption in the second week of gestation ($F_{(2, 27)}$=4.07, p<0.05). A *post-hoc* test showed that the MA gavage group consumed less food compared to the control group (Figure 5.2).

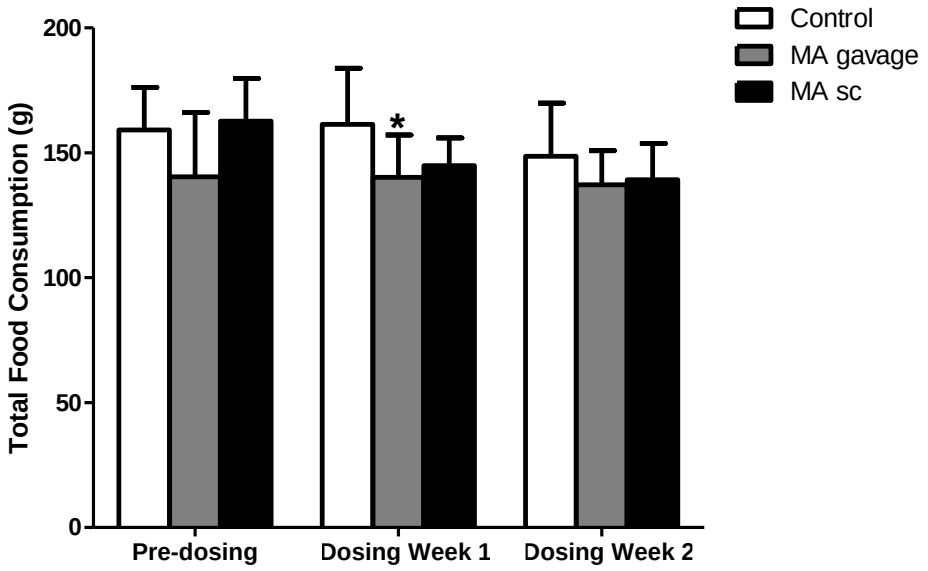

Figure 5.2: Maternal food consumption during gestation. Total food consumption from the pre-dosing and dosing periods (n=10/group). Data are expressed as Mean+SD, *p<0.05 vs. control.

Prenatal Maternal Water Consumption

A significant effect of gestation day was found ($F_{(3.06, 79.48)}$=10.94, p<0.001) with water consumption increasing slightly throughout the gestation period and then decreasing for the last few days preceding birth (GD 18 to 21). No significant effect of treatment or interaction effect of gestation day and treatment was found for water consumption or total water consumption in the prenatal period (data not shown).

5.3.2 Maternal and Litter Observations

No significant effect of treatment was found for day of birth/length of gestation period with all groups having a gestation length of 21/22 days. No significant effect of treatment was found for number of pups in a litter. A significant effect was found for number of males ($F_{(2, 38)}$=5.88, p<0.01) and females ($F_{(2, 38)}$=6.91, p<0.01) in a litter in that both MA treatment groups had more males and less females compared to the control group (data not shown). Maternal death did not occur in any treatment groups. No significant effect of treatment was found for percentage of stillborn pups, pups that were eaten or pups that died in postnatal period. No significant effect of treatment was found for total percentage of pup deaths in the postnatal period (Table 5.1). The final number of litters used for testing was ten control litters, ten MA sc litters, and ten MA gavage litters.

Drug	No. of Pups/Litter	No. of Pups Stillborn	No. of Pups Found Dead	No. of Pups Eaten	No. of Total Deaths
Control	13 ± 3	0 ± 0	0 ± 1	0 ± 0	1 ± 1
MA gavage	13 ± 3	1 ± 2	1 ± 2	1 ± 2	3 ± 5
MA sc	14 ± 2	1 ± 2	1 ± 2	1 ± 2	4 ± 4

Table 5.1: Litter observations. Litter observations at birth and in the postnatal period (n=10-17 dams/group). Data are expressed as Mean±SD.

5.3.3 Maternal Behaviour

In the observational test, a significant effect of treatment was found for active nursing ($F_{(2, 24)}$=4.40, p<0.05). A *post-hoc* test showed that the MA sc group

performed less active nursing compared to the control group. A significant effect of treatment was found for mother outside of the nest ($F_{(2, 24)}$=5.02, p<0.05). A *post-hoc* test showed that the MA sc group were outside of the nest more compared to the control group. A significant effect of treatment was found for total nursing ($F_{(2, 24)}$=4.55, p<0.05). A *post-hoc* test showed that the MA sc group performed less nursing compared to the control group. A significant effect of treatment was found for drinking ($F_{(2, 24)}$=4.00, p<0.05). A *post-hoc* test showed that the MA sc group performed more drinking compared to the control group. A significant effect of treatment was found for blanket nursing but a *post-hoc* test showed no differences between treatment groups for this parameters. No significant effect of treatment was found for the other maternal behaviours in the observational test (Table 5.2).

	Maternal activities					
Drug	**Active Nursing**	**Total Nursing**	**In nest**	**Out of nest**	**In contact with pups**	**Grooming pups**
Control	6.0 ± 2.7	6.2 ± 2.8	7.2 ± 2.7	3.4 ± 2.9	7.3 ± 2.5	1.6 ± 1.1
MA gavage	6.5 ± 1.5	6.5 ± 1.5	7.5 ± 1.6	3.0 ± 1.2	7.5 ± 1.6	1.6 ± 1.2
MA sc	3.7 ± 1.8*	3.7 ± 1.8*	4.9 ± 2.4	6.0 ± 1.9*	5.2 ± 2.3	1.2 ± 1.5

Table 5.2: Maternal behaviour in the observational test. Maternal observations in the postnatal period (n=8-10 dams/group). Data are expressed as Mean±SD for number of times the behaviour occurred, *p<0.05 vs. control.

5.3.4 Somatic Development of Offspring

Birth Weights

A significant effect of treatment was found for birth weights ($F_{(2, 54)}$=6.85, p<0.01) however a *post-hoc* test showed no difference between treatment groups for males or females. No significant effect of sex or interaction effect of treatment and sex was found (data not shown).

Pinna Unfolding

A significant effect of sex was found on PND 4 (X_1^2=12.50, p<0.05) with more females having pinna unfolded than males. A significant effect of treatment was found on PND 3 (X_1^2=53.57, p<0.001) and PND 4 (X_1^2=78.73, p<0.001) (Table 5.3). A *post-hoc* test showed that on PND 4, for males and females, all MA groups had less pups with pinna unfolded compared to their controls.

Drug	PND 3 (%)	PND 4 (%)
Male		
Control	10	70
MA gavage	10	50*
MA sc	0	40*
Female		
Control	20	90
MA gavage	0	40*
MA sc	0	50*

Table 5.3: Pinna unfolding. Percentage of pups with pinna unfolded for male and female pups (n=10/group). Data are expressed as percentage of pups with both pinna unfolded, *p<0.05 vs. relevant control.

Fur Appearance

No significant effect of sex was found on PND 3 or 4. A significant effect of treatment was found on PND 3 (X_1^2=95.24, p<0.001) and PND 4 (X_1^2=41.38, p<0.001) (Figure 5.3). A *post-hoc* test showed that, for PND 3, the male MA gavage group and both the male and female MA sc groups had less pups with fur compared to their controls.

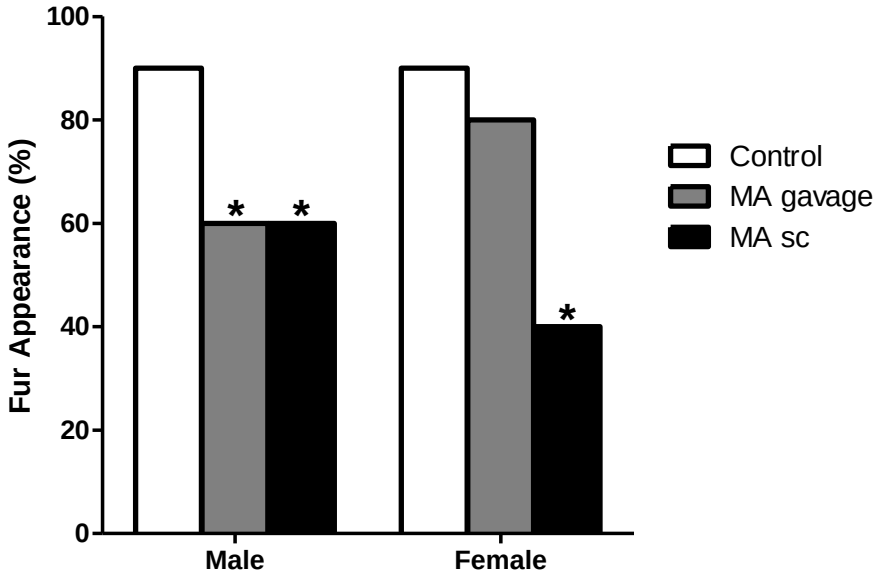

Figure 5.3: Fur appearance. Percentage of pups with fur for male and female pups on PND 3 (n=10/group). Data are expressed as percentage of pups with fur; *p<0.05 vs. relevant control.

Eye Opening

A significant effect of sex was found on PND 14 (X_1^2=35.29, p<0.001) with more females having eyes open than males but this was not seen on PND 15 or 16. A significant effect of treatment was found on PND 14 (X_1^2=117.86, p<0.001), PND 15 (X_1^2=31.58, p<0.001) and PND 16 (X_1^2=78.41, p<0.001) (Table 5.4). A *post-hoc* test showed that, for females, on PND 14 both MA groups had less pups with eyes

open compared to their controls. For females on PND 15, the MA sc group had less pups with eyes open compared to their controls. For the male MA sc group and the female MA gavage group less pups had eyes open compared to their controls on PND 16.

Drug	PND 14 (%)	PND 15 (%)	PND 16 (%)
Male			
Control	0	40	80
MA gavage	10	20	80
MA sc	0	30	40*
Female			
Control	30	50	90
MA gavage	0*	30	70*
MA sc	0*	20*	80

Table 5.4: Eye opening. Percentage of pups with eyes open for male and female pups (n=10/group). Data are expressed as percentage of pups with eyes open; *p<0.05 vs. relevant control.

Ano-Genital Distance

A significant effect of sex was found for ano-genital distance (U=0.00, p<0.001) with females having a smaller ano-genital distance compared to males. A significant effect of treatment was found also (K=44.49, p<0.001). A *post-hoc* test showed that the male MA sc group had smaller ano-genital distances compared to their controls (Table 5.5). No significant interaction effect of sex and treatment was found.

Drug	Ano-genital distance (mm)
Male	
Control	3.99 (3.56 - 4.18)
MA gavage	3.57 (2.40 - 3.91)
MA sc	3.10 (2.94 – 3.45)*
Female	
Control	2.04 (1.81 – 2.21)
MA gavage	1.84 (1.50 – 2.06)
MA sc	1.66 (1.45 – 2.35)

Table 5.5: Ano-genital distance. Ano-genital distance (mm) for male and female pups on PND 3 (n=10/group). Data are expressed as Median and Interquartile range; *p<0.05 vs. relevant control.

Body Length

No significant effect of sex or interaction effect of sex and treatment was found. A significant effect of treatment was found ($F_{(2, 54)}$=7.34, p<0.01). A *post-hoc* test showed that the male MA sc group had smaller body lengths compared to their controls (Table 5.6).

Drug	Body length (mm)
Male	
Control	69.0 ± 3.0
MA gavage	68.0 ± 2.8
MA sc	65.0 ± 3.8*
Female	
Control	67.7 ± 2.7
MA gavage	66.0 ± 2.9
MA sc	64.5 ± 2.7

Table 5.6: Body length. Body length (mm) for male and female pups on PND 7 (n=10/group). Data are expressed as Mean±SD, *p<0.05 vs. relevant control.

Neonatal Body Weight

A significant effect of postnatal day was found ($F_{(1.68, 50.44)}$=1321.15, p<0.001) with all groups gaining weight as time progressed. No significant effect of treatment, sex or interaction effect of sex and treatment was found (data not shown). No significant effect of treatment, sex or interaction effect of sex and treatment was found for total body weight gain (PND 1-15) (data not shown).

5.3.5 Behavioural Testing of Offspring

Surface Righting

A significant effect of sex was found on PND 2 (X_1^2=12.50, p<0.001), PND 3 (X_1^2=50.51, p<0.001), PND 4 (X_1^2=22.22, p<0.001) and PND 5 (X_1^2=22.22, p<0.001) with males performing better than females each day. A significant effect of treatment was found on PND 2 (X_1^2=66.03, p<0.001), PND 3 (X_1^2=150.00, p<0.001), PND 4 (X_1^2=112.09, p<0.001) and PND 5 (X_1^2=85.71, p<0.001) (Table 5.7). A *post-hoc* test showed that for males, the MA sc group on each testing day, and the MA gavage group on PND 2 and 4, had fewer pups that could reach the criterion of 10 s compared to the control group. For females, the MA sc group on PND 2 and 5, and the MA gavage group on PND 4 had fewer pups that could reach the criterion of 10 s compared to the control group.

Drug	PND 2 (%)	PND 3 (%)	PND 4 (%)	PND 5 (%)
Male				
Control	90	80	100	100
MA gavage	70*	70	70*	90
MA sc	50*	10*	50*	50*
Female				
Control	70	30	80	80
MA gavage	60	30	50*	70
MA sc	40*	60*	40*	70

Table 5.7: Surface righting. Surface righting for male and female pups on PND 2, 3, 4 and 5 (n=10/group). Data are expressed as percentage of pups that complete test in less than 10 s; *p<0.05 vs. relevant control.

Inclined Plane

No significant effect of sex was found on PND 9 or 11. A significant effect of treatment was found on PND 9 (X_1^2=132.63, p<0.001) and PND 11 (X_1^2=33.15, p<0.001). For males on PND 9, the MA gavage group had less pups that could reach the criterion of 15 s compared to the control group. For females, on PND 9 the MA gavage and MA sc, had less pups that could reach the criterion of 15 s compared to the control group. On PND 11 the female MA gavage and MA sc groups, had less pups that could reach the criterion of 15 s compared to the control groups (Figure 5.4).

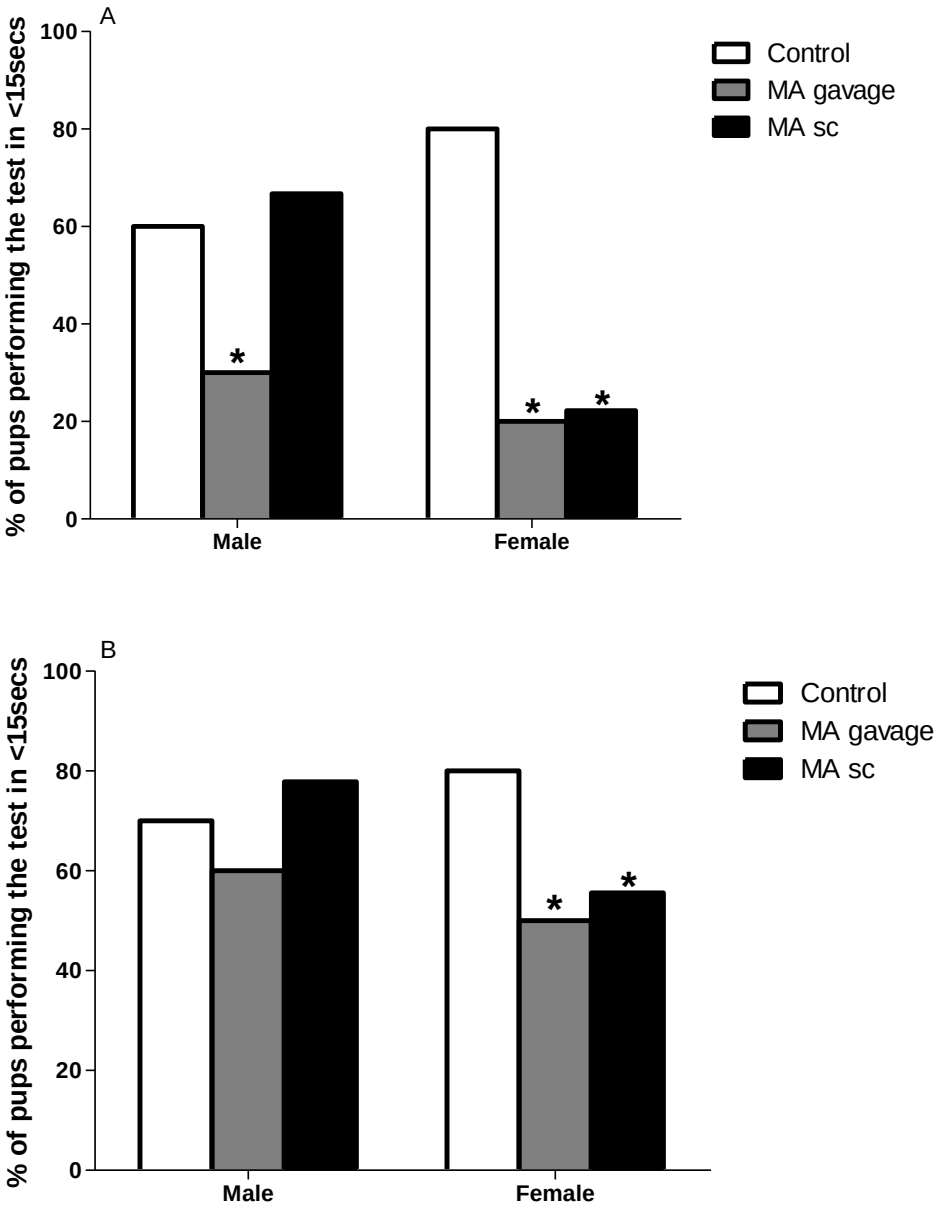

Figure 5.4: Inclined plane. Inclined plane test for male and female pups on (A) PND 9 and (B) PND 11 (n=10/group). Data are expressed as percentage of pups that complete test in less than 15 s; *p<0.05 vs. relevant control.

Forelimb Grip

A significant effect of sex was found on PND 14 (X_1^2=33.33, p<0.01) with males performing better than females but this was not found on PND 17. A significant effect of treatment was found on PND 14 (X_1^2=97.43, p<0.001) and PND 17

(X_1^2=27.12, p<0.001). A *post-hoc* test showed that on PND 14, for males, the MA gavage and MA sc groups had less pups that could perform the task for more than 10 s compared to the control group. On PND 17, the male MA gavage and female MA sc groups had less pups that could perform the task for more than 10 s compared to the control groups (Figure 5.5).

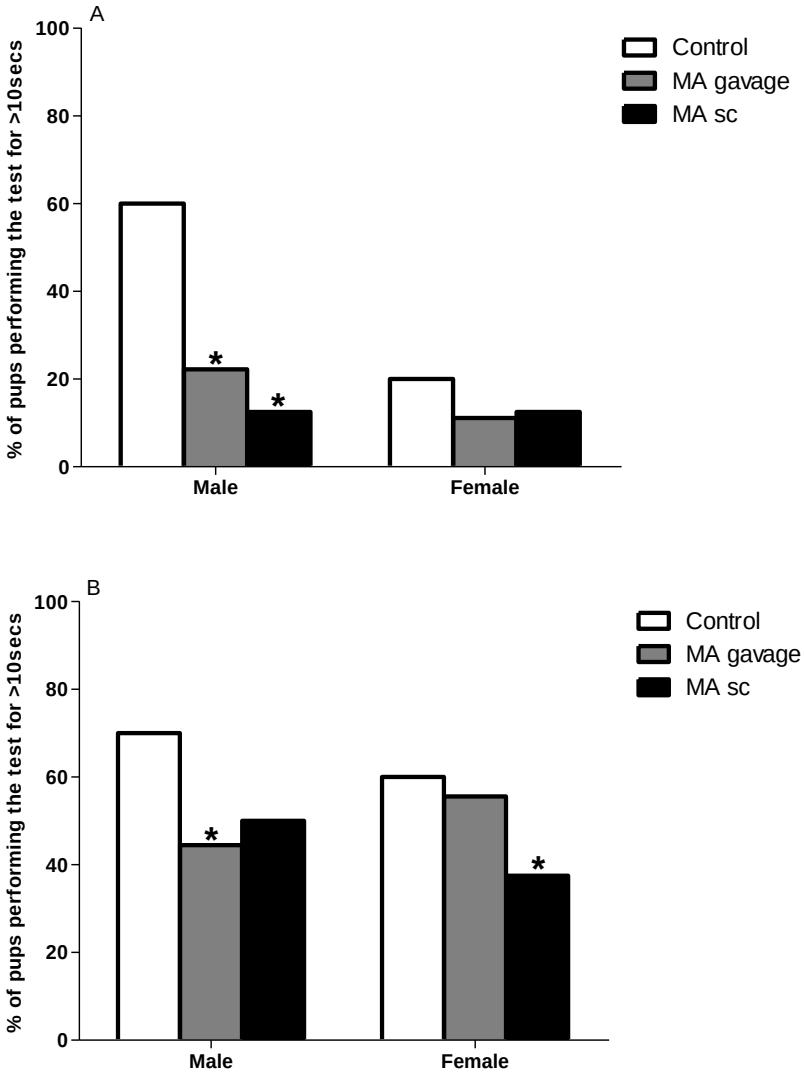

Figure 5.5: Forelimb grip. Forelimb grip for male and female pups on (A) PND 14 and (B) PND 17 (n=10/group). Data are expressed as percentage of pups that could perform the task for more than 10 s; *p<0.05 vs. relevant control.

5.4 Discussion

The aim of this study was to determine if the route of administration for MA during pregnancy in rats is a contributing factor to the neurodevelopmental and behavioural alterations observed in the rat offspring. The present study confirms that prenatal MA exposure at a pharmacological dose has adverse effects on the developing offspring and can delay neurodevelopment and alter behaviour in the rat offspring. The results illustrate that the route of administration of MA during pregnancy in rats does have an influence on the outcome of the offspring with the subcutaneous route having a greater effect on the parameters assessed during the neonatal period. The results highlight three principal findings: 1. Altered maternal behaviour after sc MA, 2. Augmented effects on previously established MA findings after sc MA (most likely due to increased potency with this route), 3. Unique attributes of sc MA on neonatal outcome that have not been previously reported for oral MA.

The first objective of this study was to determine if prenatal MA exposure given orally or via sc injection had any consequences on maternal parameters. With regard to effects of MA exposure on the dams, our previous findings with this dose of MA, given orally, is that MA administered prenatally reduces body weight gain and food intake in the first days of drug administration and that these effects are transient and only observed at the start of the MA dosing period (McDonnell-Dowling and Kelly, 2015a). In the present study we found the same effects of prenatal MA but when given subcutaneously the effect is greater and the dam's weight gain is below that of the controls as well as that of the gavage-treated dams. This is in agreement with other studies that have given MA subcutaneously at similar doses (Cho *et al.*, 1991) but is in contrast with other studies using sc with higher MA doses (Slamberova *et*

al., 2005a, Pometlova *et al.*, 2009, Malinova-Sevcikova *et al.*, 2014). The MA treatment groups differ however, in that the reduction of weight gain in the gavage dams can be attributed to reduced food intake, a common occurrence (Kraeuchi *et al.*, 1985), while this did not reach significance for the sc dams. Therefore it may be attributed to increased locomotor activity. The increased amount of MA available in the brain may generate a greater behavioural effect causing increased locomotor activity but unfortunately this was not examined in the present study and may warrant further investigation. Regardless, by the last week of the dosing period both body weight and food consumption are comparable to the control dams for both MA treatment groups.

Examining maternal behaviour in the postnatal period, sc MA was shown to alter behaviour of the mother towards the pups. Traditionally, the observation test is performed daily from birth until PND 12. For the present study, a 'snapshot' of maternal behaviour was taken and this test was performed only on PND 10. Nonetheless, the MA gavage mothers exhibited similar behaviour towards their offspring as the control mothers in the observational test including time spent in nest, time with pups, nursing of pups, grooming of pups and other behaviours such as nest building and self-care. This suggests that prenatal oral MA has no substantial influence on the mother's behaviour and suggests that any outcomes observed in the offspring are due to the prenatal drug exposure rather than maternal alterations. On the other hand, the MA sc mothers exhibited altered behaviour towards their pups. Mothers did not tend to the pups or care for the pups as much in comparison to controls. This was shown by increased time spent outside of the nest and less time spent nursing and actively nursing the pups. It has been previously shown that maternal behaviour can be impaired by MA treatment at the higher dose of 5 mg/kg

sc (Slamberova *et al.*, 2005a, Slamberova *et al.*, 2005b). Altered behaviours that have previously been reported include less time spent in the nest, time spent in contact with the pups and time spent grooming the pups. These maternal changes towards the pups can therefore have a significant impact on offspring development. Consequently, any effects found on the offspring may not be due to the drug treatment alone and may in fact be due to altered maternal behaviour.

The second objective of this study was to determine if prenatal MA exposure given via different methods of administration has an effect on somatic and behavioural development in the offspring. The somatic development of the pups highlighted many developmental delays after exposure to MA. Pinna unfolding, fur appearance and eye opening all mark significant milestones in offspring development, all of which were delayed by prenatal MA exposure. Both MA treatments delayed these somatic development parameters and this is in agreement with other studies for both oral gavage (McDonnell-Dowling *et al.*, 2014, McDonnell-Dowling and Kelly, 2015) and sc routes (Cho *et al.*, 1991, Pometlova *et al.*, 2009, Hruba *et al.*, 2009b). Moreover, looking more closely at the results for somatic development it is apparent that sc MA actually amplifies these developmental delays in that fur appearance and eye opening were more severely affected when compared to MA gavage. The behavioural and reflex ontogeny results, such as surface righting, inclined plane test and forelimb grip, also indicated the same pattern of outcomes. Each test reflected behavioural deficits in the offspring for both MA treatment groups but once more the deficits were exacerbated in the MA sc offspring. Less of the gavage MA pups were able to complete the surface righting task for one or two of the postnatal testing days, for females and males respectively, whereas less of the sc MA pups were able to

complete the task for three or four of the days, for females and males respectively. The inclined plane test revealed similar behavioural shortfalls in the female offspring for both MA groups given that less female pups could perform this task regardless of MA route on both testing days. In the forelimb grip test, the female pups only displayed behavioural alterations on the second day of testing and these observations were only seen in the sc MA pups. Underperformance in the male offspring was seen for both MA groups on the first testing day in that less male pups could perform this task in both MA groups (although again this was poorer in the sc pups). The difference in outcomes between these exposures can be rationalised by the level of MA that is available in the brain and the blood of the mother and therefore available to the foetuses. Thus the impact at a crucial period of development is more affected by the increased concentration of the drug that the foetus is exposed to. The clearance of MA in late gestation regardless of the route of administration is reduced and so the half-life of the drug is increased at the time just before partition occurs. Correspondingly, it should be noted that development of the cerebellum (vital for balance and co-ordination) in rats does not occur until the end of the gestation period. The increased amount of MA available at this crucial time in brain development may therefore explain the pronounced deficits in behavioural tests compared to somatic developmental parameters.

The present study also found additional developmental deficits for sc MA that are not observed with oral MA. Development of somatic parameters including ano-genital distance and body length were inhibited by MA sc treatment only and this was only observed in the male pups. The sexual maturation of the male pups was significantly different in that the ano-genital distance was smaller in the sc pups

compared to the control pups. This effect in the early postnatal period on the male pups has not been reported before for higher doses of MA sc (5 mg/kg sc, (Pometlova *et al.*, 2009)), however it is unclear exactly how this measure was taken and so a difference in protocols between our studies may explain this result. Unfortunately, no other literature exists to compare prenatal MA effects on ano-genital distance. To the best of our knowledge, this parameter has not been investigated for other amphetamines or drugs of abuse. Other substances that have shown reduced ano-genital distance include di-butyl phthalate (500 mg/kg/day GD 13-20 via gavage), paracetamol (150, 250 and 350 mg/kg/day GD 13-21 via gavage) and acetylsalicylic acid (150, 200 and 250 mg/kg/day GD 13-21 via gavage) (Wolf *et al.*, 1999, Kristensen *et al.*, 2011, Lourenco *et al.*, 2014). Decreased or delayed development of the ano-genital distance is an indicator of reduced intrauterine androgen levels (Welsh *et al.*, 2008, Kristensen *et al.*, 2011). In rats (and all mammals), the reproductive system is initially undifferentiated for males and females and "masculinization" for males is driven by hormones (primarily testosterone) produced by the fetal testis (Jost, 1972, Welsh *et al.*, 2008). Impaired fetal levels of androgens therefore interfere with this masculinization process and can result in disorders of sexual differentiation (Hughes and Deeb, 2006, Welsh *et al.*, 2008). It was considered that this effect may be related to retarded development of the pups as body length was also smaller for the male sc MA pups compared to controls, and this was not seen in females or the oral MA groups. However, we also investigated body weight gain during the neonatal period and this was shown to be unaffected with all male sc MA pups weighing the same as control pups. Therefore the smaller male ano-genital distances and body lengths are developmental retardations and not repercussions based on the offspring being under weight. The

smaller male ano-genital distance may be due to a delay in ‘‘masculinization’’ caused by a decrease in intrauterine androgen levels. It is unknown if this effect is still present in later life and unfortunately it was not feasible to measure this again in the later postnatal period. However, a study by Cho *et al.* (1991) showed that testes descent (another measure of sexual maturation) in the third postnatal week in male pups was delayed at a similar dose of MA (3 mg/kg) given during pregnancy via sc injection. This therefore warrants further investigation. Body length has not been previously investigated in neonatal studies examining prenatal MA exposure via sc but our previous findings with oral MA exposure at the same dose showed that body length was also unaffected in male and female offspring (McDonnell-Dowling and Kelly, 2015a) therefore, this is also a unique attribute found for prenatal sc MA.

The absence of these attributes in female pups is unexpected as it is thought that females have a greater sensitivity to MA toxicity. For example, a sex difference in MA pharmacokinetics has been reported previously and higher MA levels are seen in female plasma and brains compared to males (Rambousek *et al.*, 2014). Sex dissimilarities to toxicity of drugs such as MA can be explained by the differences in concentrations of cytochrome P-450 and other hormones, as drugs are more slowly eliminated in female rats (Baba *et al.*, 1988, Kato and Yamazoe, 1992, Mugford and Kedderis, 1998). The increased sensitivity and perhaps vulnerability of female offspring to prenatal MA is noteworthy and this warrants that females are included for further investigation to see the potential sex difference in MA toxicity. Although in the present study we found that males were more negatively affected by MA exposure this was not the case in our previous studies reporting no sex difference in neurodevelopmental alterations with a slight trend for females being more severely affected (McDonnell-Dowling *et al.*, 2014, McDonnell-Dowling and Kelly, 2015a).

In conclusion, this study highlights that the route of administration should be considered when interpreting preclinical studies investigating prenatal MA exposure. Moving into the future, it stresses the value of choosing a route of administration in preclinical studies that is most commonly associated with use in humans.

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Chapter 6:

Baseline behaviour and responses to psychopharmacological challenges in adulthood after methamphetamine exposure during pregnancy or lactation in rat offspring

Abstract

With the increasing number of pregnant females abusing MA there has been a parallel increase in the number of clinical and preclinical studies investigating the consequences of such exposure. Yet, there is still a need for studies which examine the long-term consequences of this in adulthood. To date the preclinical literature has deviated from the clinical scenario with regards dose of drug, exposure timing, exposure frequency and routes of administration. Thus, the aim of this study was to use a clinically relevant animal model to determine if the administration of MA during pregnancy or lactation at pharmacological doses has long-lasting effects on neurodevelopment and behaviour in the rat offspring. Pregnant Sprague-Dawley dams (n=8-12 dams/group) received MA (0.625, 1.25, 2.5 or 3.75 mg/kg) or control (distilled water) via oral gavage or sc injection during the gestation and/or postnatal period in a variety of dosing scenarios. A range of well-recognised behavioural parameters were examined in the offspring in adulthood (PND 56, 85 and 113) and psychopharmacological challenges were also evaluated. This study showed that alterations in behaviour are still present in adulthood after prenatal MA exposure. The main findings in this study are that prenatal MA at low doses results in decreased anxiety-like behaviour in the elevated plus maze and increased depressive-like behaviour in the forced swim test. These behavioural effects were most defined in the prenatal 1.25 and 2.5 mg/kg MA and the 3.75 mg/kg MA groups, respectively. No significant effects were observed for the psychopharmacological challenges. Overall, this highlights the long-term outcomes for offspring when prenatally exposed to MA and emphasises the difference in outcome when exposed to different doses of MA.

6.1 Introduction

The abuse of MA has become an increasing problem worldwide. This can be illustrated by statistics from a national survey in the US which reported that in 1995 approximately 21,000 people had begun use of this drug (National Survey on Drug Use and Health, 2005). Moving forward to 1999, there was approximately 186,000 new users of MA and in 2003 there was approximately 720,000 new users. This alarming trend is also observed in pregnant females that are opting to abuse MA over other drugs of abuse such as cocaine. Awareness of the consequences of such exposure during pregnancy on the offspring is growing both clinically and preclinically. Children aged 3-4 years that have been exposed to MA *in utero* have shown alterations in white matter maturation (Cloak *et al.*, 2009), lower striatal volumes (Berman *et al.*, 2008) and deficits in inhibitory control performance (Derauf *et al.*, 2012). At the age of 7.5 years, children who are prenatally exposed to MA have increased cognitive problems, an effect still observed when controlled for home life experience (Diaz *et al.*, 2014). Children up to the age of 16 years that have been exposed to MA *in utero* show smaller subcortical volumes, lower long-term spatial memory, lower visual motor integration, lower verbal memory and lower attention (Chang *et al.*, 2004). Yet, there is a paucity of studies that have followed the development of these children in later life. This is most likely due to the fact that these cohorts of children are still of a young age and have not yet reached adolescence or adulthood. This trend of MA abuse in pregnancy is quite a recent phenomenon and so it is still unclear if any long-term effects are associated with prenatal MA abuse. Consequently, preclinical studies have aimed to elucidate this and have investigated if indeed there are any longstanding developmental delays. To date, the preclinical literature has investigated alterations in locomotor activity,

anxiety, pain, memory and learning. It has been shown that, in adulthood, prenatal MA exposure increases anxiogenic behaviour in male rats (Slamberova *et al.*, 2015), increases compulsivity, impulsivity and motivation for reward in male and female mice (Lloyd *et al.*, 2013) and increases amphetamine-seeking behaviour as well as morphine-induced drug-seeking behaviour in male rats (Slamberova *et al.*, 2012a). Exposure to MA prenatally also results in changes in NMDAR subunit expression in male adult rats but not in male adolescent rats (Vrajová *et al.*, 2014). Nonetheless, as previously mentioned there is a great deal of variation among these preclinical behavioural tests (McDonnell-Dowling and Kelly, 2015). Variations in performance of behavioural tests, exclusion of female offspring from testing and again deviations from the clinical scenario make these previous preclinical results difficult to interpret and to extrapolate clinically. In order to more comprehensively assess the long-term effects of prenatal or postnatal MA exposure in offspring, a range of behavioural endpoints need to be assessed in order to capture a thorough picture of long-term development. Responsiveness to other drugs in later life may also reveal other alterations in adult development. Therefore, the aim of this study was to create a behavioural profile of adult male and female rats exposed to MA during gestation or lactation. The study aimed to investigate alterations in adult behaviour after a range of clinical scenarios including: prenatal exposure to different doses of MA, postnatal exposure to MA, intermittent or acute prenatal exposure to MA and gavage vs sc prenatal exposure. Another aim of the study was to investigate alterations in adult behaviour after psychopharmacological challenges in adulthood following prenatal exposure to MA.

6.2 Materials and Methods

Experiment 1

The aim of experiment 1 was to obtain a behavioural profile in adulthood of rats exposed to MA during gestation, at a range of doses (offspring continuing on from chapter 2).

Experiment 2

The aim of experiment 2 was to obtain a behavioural profile in adulthood of rats exposed to MA during gestation or lactation (offspring continuing on from chapter 3).

Experiment 3

The aim of experiment 3 was to obtain a behavioural profile in adulthood of rats exposed to MA intermittently or acutely during gestation (offspring continuing on from chapter 4).

Experiment 4

The aim of experiment 4 was to obtain a behavioural profile after psychopharmacological challenges in adulthood of rats exposed to MA during gestation via gavage or sc (offspring continuing on from chapter 5).

6.2.1 Animal Housing

Adult male (275–325 g, approx. 3.5-4 months old) and female (250–325 g, approx. 3.5-4 months old) Sprague-Dawley rats were used for this study. All females were bred in-house; all males came from Charles River (Kent, U.K.) and animals were habituated for one week from arrival. After mating, all female rats were housed singly in plastic bottom cages with appropriate bedding material (Gold flake sawdust, Harlan, UK or Pellets, 3Rs™, UK) and additional nesting materials

(unbleached cotton and Nesteldown bedding, Petworld, Galway). All animals were maintained under standard laboratory conditions under artificial 12-h light/dark cycle (lights on from 08:00h) and temperature was maintained at 20-24^{o}C with relative humidity at 35-60%. Food and water were provided *ad libitum*. Following littering, the rat pups remained with their biological dams until PND 21, at which point the pups were weaned. Cross-fostering was not employed in this study in order to mimic clinical scenarios but also to ensure that active drugs present in the MA mother are not passed onto the control pups via breast milk or urinary and faecal excretions (McDonnell-Dowling and Kelly, 2015b). The pups stayed with their siblings until PND 28, at which point they were then separated by sex; littermates of the same sex remained together throughout adulthood. All experiments were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway (12/NOV/07) and in compliance with the European Communities Council directive 86/609.

6.2.2 Mating

A male:female ratio of 1:3 for mating was used. Each cage of female rats (3) were housed overnight with one sexually mature male rat. At the beginning of the light phase the following morning, vaginal smears were obtained from all females to check for the presence of sperm. All smears were examined under a light microscope.

6.2.3 Gestation Period and Deliveries

The expected day of delivery (birth) in rats is GD 21-22. Pregnant females were checked daily; offspring in each litter were checked and counted daily in the week

after delivery to monitor for pup mortalities. The pups were randomly culled (using a random number generator) to 10 per litter on PND 1 with a litter ratio of 50:50 of males:females whenever possible. One or two males and females were selected for testing from each litter for each behavioural test/testing time point in order to avoid litter effects.

6.2.4 Drug Treatment

Methamphetamine HCl was purchased from Sigma Aldrich (St. Louis, MO; M8750). Rats were assigned randomly to control or MA-treated groups based on body weights and likelihood of pregnancy. The MA was given at a volume of 1 ml/kg and controls received the VEH alone, i.e. 1 ml/kg distilled water. The doses of MA selected compare to clinically relevant doses in the human scenario and the use of an allometric scale takes into account the body weight and body surface area of a pregnant human versus a pregnant rat (Reagan-Shaw *et al.*, 2008), therefore maintaining the viability of the project and its comparison to clinical situations. Oral gavage was used in all experiments as this represents the most common route of MA administration in humans (U.S. Department of Health and Human Services, 2006), but has been disregarded in preclinical investigations. For the drug challenges in adulthood desipramine HCL was purchased from Sigma Aldrich (Wicklow, Ireland; D3900), d-amphetamine sulphate was purchased from Sigma Aldrich (St. Louis, MO; A5880), methamphetamine HCl was purchased from Sigma Aldrich (St. Louis, MO; M8750) and diazepam (Diazemuls) was purchased from the local pharmacist (University pharmacy, Galway, Ireland).

Experiment 1: Dose Response

For this experiment 72 females and 24 males were acquired. The doses of MA used were 0.625, 1.25 and 2.5 mg/kg. For MA or control treatments, the dams were dosed both via oral gavage once daily at 14.00h from GD 7 until 21 (time of birth).

Experiment 2: Prenatal and Postnatal

For this experiment 52 females and 12 males were acquired. The dose of MA used was 3.75 mg/kg. For MA or control treatments, the dams were dosed via oral gavage once daily at 14.00h from GD 7 until PND 21 (time of weaning). During the gestation period two groups of dams received VEH and one group received MA. During the postnatal period, one group of dams receiving VEH continued to take VEH and the other was switched to MA, and the group of dams receiving MA was switched to VEH.

Experiment 3: Intermittent Exposure

For this experiment 40 females and 12 males were acquired. The dose of MA used was 3.75 mg/kg. Three treatment groups were determined: during the gestation and postnatal periods one group of mothers received VEH (control); the next group of mothers was given a single dose of MA (acute) on GD 21 and on all other days was given VEH; the last group of mothers was given MA twice in each gestational week (intermittent) on GD 6 and 7, 13 and 14, 20 and 21 and on all other days was given VEH.

Experiment 4: Route of Administration

For this experiment 59 females and 20 males were acquired. The dose of MA used was 3.75 mg/kg. For MA or control treatments, the dams were dosed both via oral gavage and sc injection once daily at 14.00h from GD 7 until 21 (time of birth). Subcutaneous injection was used in this study as this represnets the most common

route of MA administration in preclinical studies. Three treatment groups were determined: during the gestation period one group of mothers received VEH (control) via gavage and sc routes; the next group of mothers was administered MA via oral gavage and VEH via sc injection; the third group of mothers was administered MA via sc injection and VEH via oral gavage.

6.2.5 Behavioural Testing

A variety of behavioural tests were performed on the selected rats. Specific PNDs were selected for the behavioural tests and these began at 08.00h on the appropriate PND. At the time of testing, the rats were removed from the home cage and placed into the arena. All testing occurred in novel cage rooms outside the home cage room except for the home cage activity (HCA). Unless stated otherwise, the rat was placed back into the home cage after testing was completed. All experimenters were blind to the treatment condition of the animal during testing.

Elevated Plus Maze

The EPM consists of a plus shape with two open arms (50 cm×13 cm), two enclosed arms (50 cm×13 cm) and a central platform (10 cm×13 cm) made of black polycarbonate plastic. Each arm is supported by a sturdy plastic leg and is raised 46 cm above the floor. The two enclosed arms have high walls (30 cm in height) on the sides and ends. To avoid shadows over the arms one 100W bulb is placed above each of the open arms. The intensity of light was set at 80 lux in the open arms and 35 lux in the closed arms for experiments 1 and 2 and at 120 lux in the open arms and 55 lux in the closed arms for experiment 3 and 4. Behaviours were recorded by a camera above the EPM linked to a DVD/DVR recorder in the same room. Ethovision video tracking software was used to track and score behavioural measures; duration

of time spent in each zone (centre, open and closed arms), frequency of entries into each zone (centre, open and closed arms), total distance moved and velocity of movements within the EPM. At the time of testing, the selected rats were taken directly from their home cages and placed in the centre of the EPM facing one open arm. The test lasted for 5 min after which the animal entered straight into the open field test, where applicable. The apparatus was cleaned between each animal. For drug challenges, a diazepam (DZP) dose of 1 mg/kg sc at a volume of 1 ml/kg was administered. Thirty min after drug administration the animal was placed into the EPM as above and then sacrificed after the test was completed.

Open Field

The OF apparatus consists of a white, circular base (75 cm diameter) surrounded by a wall (43 cm in height). The base of the OF is divided into 10 cm squares by black lines. To avoid shadows in the OF four 100W bulbs are placed above the OF with the intensity of light set at 190-210 Lux. Behaviours were recorded by a camera above the OF linked to a DVD/DVR recorder in the same room. Ethovision video tracking software was used to track and score behavioural measures; duration of time spent in each zone (inner and outer), frequency of entries into each zone (inner and outer), total distance moved and velocity of movements within the OF. The selected rats were taken directly from the EPM and placed in the centre of the OF. The test lasted for five min after which the animal was sacrificed. After each test the OF was cleaned before the next animal. No drug challenges were used in this test.

Forced Swim Test

The forced swim test (FST) apparatus consists of four Pyrex cylinders (45 x 20 cm) lined up side by side and separated by black polycarbonate plastic walls. The cylinders are raised 75 cm above the floor. The apparatus was illuminated by a

100W bulb placed in front of the FST with the intensity of light set at 30 lux. Each cylinder was filled to 30 cm from the base and water temperature was between 23-26ºC. On the habituation day, the selected rats were taken directly from their home cages and placed into the FST. The rats were left for a pre-swim period of 15 min. Twenty-four hours later the same rat was then re-exposed to the FST for a period of 5 min. After each test, the FST was emptied and refilled with clean water before the next animal. Behaviours were recorded by a camera in front of the FST linked to a DVD/DVR recorder in the same room. Ethovision video tracking software was used to manually track and score behavioural measures; duration of time spent swimming, climbing and immobile within the FST. When the test was complete, the animal was sacrificed. For drug challenges, the preswim was used as an indication of the baseline behaviour (Cryan *et al*., 2005, Lucki, 1997) and the rat was administered subacute desipramine (DMI, 10 mg/kg sc) at a volume of 2 ml/kg, 24 h, 5 h and 1 h before the second FST exposure (swim).

Morris Water Maze

The MWM apparatus consists of a tank measuring 2 m in diameter and filled to a depth of 0.3 m with water. The water temperature was between 23-25ºC. An escape platform was located in one quadrant of the tank (SW) and was submerged (2 cm) below the surface of the water (i.e. water filled to 0.32 m) for the acquisition trials. Large geometric shapes (printed in black and white on A4 paper) were placed around the maze to provide visual cues. These cues were held in constant spatial relations throughout the experiments. The apparatus was illuminated by four 100W bulbs placed above the MWM with the intensity of light set at 25 lux. On acquisition day 1, the selected rats were taken directly from their home cages and placed into the MWM from varied starting points from Nature Protocols (Vorhees and Williams,

2006), so that it was facing the wall of the pool. The rat was allowed to swim around until it found the platform with a max duration of 120 sec. Blocks of four trials were presented to each rat, one block per day (this is the acquisition phase and lasts for 4 days). On reaching the platform, each rat was allowed to remain there for 10 sec before being removed from the maze, dried gently using a cotton towel and placed in a recovery cage. If a rat failed to locate the platform within 120 sec, it was guided there by the experimenter and left here for 10 sec. Once the rat was placed in its recovery cage, a timer was set. When the same rat was then removed for its next trial, the inter-trial interval on the timer was recorded. In the acquisition phase, the platform was hidden in the southwest pool quadrant; in the probe trial the platform was absent and the rats were released from a novel release point (NE). Behaviours were recorded by a camera above the MWM linked to a DVD/DVR recorder in another room. Ethovision video tracking software was used to track and score behavioural measures; distance and velocity travelled within the MWM and time spent in SW quadrant on probe day (testing day). Performance was also rated on latencies to find the platform. No drug challenges were used in this test.

Home Cage Activity

The HCA apparatus consists of a rack with four/eight individual places separated by black polycarbonate plastic walls. The home cage consists of a plastic bottom cage (42 × 25.5 × 13 cm) that contains a black Perspex plate on the base (inserted the night before testing). The individual places were illuminated by 100W bulbs placed above the cage with the intensity of light set at 55 lux. On the day of testing, the selected rats were placed into the HCA rack in their home cage. The rats were left for a period of 60 min. Behaviours were recorded by individual cameras above the HCA linked to a DVD/DVR recorder in the same room. Ethovision video tracking

software was used to track and score behavioural measures; distance, immobility and velocity travelled within the HCA. When the test was complete, the animal was sacrificed. For drug challenges in experiement 1, after the initial 60 min in the HCA rats were administered a MA challenge dose (1 mg/kg sc) at a volume of 1 ml/kg and rats were then immediately placed back into the HCA rack in their home cage for a further 120 min. For drug challenges in experiment 4, the rats were administered an AMP challenge dose (1 mg/kg sc) at a volume of 1 ml/kg immediately before the 60 min exposure to the HCA.

6.2.6 Statistical Analysis

All figures representing the data from the testing period were constructed using Graphpad Prism 5.0. The data were then analysed using the statistical package SPSS 20.0 or 22.0. Data were firstly assessed to determine if it displayed normality of distribution and homogeneity of variance (Shapiro–Wilks and Levene's test $p>0.05$). The data were also assessed to determine if it displayed sphericity (Mauchly's test $p>0.05$) however, if this failed then the Greenhouse–Geisser correction was used (i.e. degrees of freedom corrected). For the parametric data, tests used included: repeated measures ANOVA to compare the overall effect for related data, Two-Way ANOVA to compare the effect of treatment groups and sex, One-Way ANOVA and Student-Newman Keuls *post-hoc* tests were used to define where the significance lay. For the non-parametric data tests used included: Friedman's ANOVA by Ranks to compare the overall effect for related data, Wilcoxon Match-Pairs test to compare the effect of time, Kruskal-Wallis test to compare the effect of treatment groups and Mann-Whitney U tests were used to define where the significance lay. The level of

significance was set at $p<0.05$ for all parameters except when the Bonferroni correction was employed when it was set at $p<0.01$ (due to multiple comparisons).

6.3. Results

6.3.1 Experiment 1: Dose Response

Elevated Plus Maze (PND 56)

A significant effect of sex was found for distance ($F_{(1, 60)}=9.94$, $p<0.01$), velocity ($F_{(1, 60)}=10.14$, $p<0.01$) and OAT ($F_{(1, 60)}=5.80$, $p<0.05$) with males moving less, at a slower speed and spending less time in the open arms compared to females (data not shown). No significant effect of sex was found for OAE. No significant effect of treatment or interaction effect of sex and treatment was found for any of the parameters.

Open Field (PND 56)

A significant effect of sex was found for distance ($F_{(1, 63)}=4.41$, $p<0.05$) and velocity ($F_{(1, 63)}=4.41$, $p<0.05$) with males moving less and at a slower speed compared to females. A significant effect of treatment was found for distance ($F_{(3, 63)}=4.33$, $p<0.01$) and velocity ($F_{(3, 63)}=4.51$, $p<0.01$). A *post-hoc* test showed that there were no differences for the treatment groups compared to the control (data not shown). No significant interaction effect of sex and treatment was found for any of the parameters.

Forced Swim Test (PND 56)

A significant effect of treatment was found for time spent swimming ($F_{(3, 61)}$=2.95, p<0.05) and time spent immobile ($F_{(31, 61)}$=2.92, p<0.05). A *post-hoc* test showed that there were no differences for the treatment groups compared to the controls (data not shown). No significant effect of treatment was found for time spent climbing. No effect of sex or interaction effect of sex and treatment was found for any of the parameters.

Elevated Plus Maze (PND 85)

A significant effect of treatment was found for distance (K=21.47, p<0.01), velocity (K=21.48, p<0.01), OAT (K=18.66, p<0.01) and OAE (K=20.95, p<0.01) (Table 6.1). A *post-hoc* test showed that the 0.625 mg/kg MA females had a smaller distance travelled and slower velocity and the 1.25 mg/kg and 2.5 mg/kg MA females had a higher OAT and OAE compared to the control females. A significant effect of sex was found for distance (U=275.00, p<0.001), velocity (U=272.00, p<0.001) and OAT (U=344.50, p<0.01) with males moving less, at a slower speed and spending less time in the open arms compared to females. No significant effect of sex was found for or OAE.

Drug	Distance moved (cm)	Velocity (cm/s)	Open Arm Time (%)	Open Arm Frequency (%)
Male				
Control	1154 (1003-1481)	4 (3-5)	13 (2-25)	34 (21-41)
0.625mg	1328 (1099-1500)	4 (4-5)	4 (0-12)	13 (6-21)
1.25mg	1052 (939-1382)	4 (3-5)	19 (6-29)	33 (27-40)
2.5mg	1317 (1044-1384)	5 (4-5)	16 (9-30)	32 (25-51)
Female				
Control	1445 (1416-1659)	5 (5-6)	12 (3-23)	33 (27-46)
0.625mg	1315** (1242-1407)	4* (4-5)	5 (3-12)	24 (15-26)
1.25mg	1516 (1041-1861)	5 (4-6)	24** (5-39)	43** (32-51)
2.5mg	1604 (1390-1896)	5 (5-6)	21* (14-30)	41** (35-47)

Table 6.1: Elevated plus maze. Parameters of the EPM for males and females on PND 85 (n=13-20/group). Data are expressed as Median and Interquartile range; $^{**}p<0.01$, $^{*}p<0.05$ vs. relevant control.

Open Field (PND 85)

No significant effect of sex, treatment or interaction effect of sex and treatment was found for any of the parameters (data not shown).

Morris Water Maze (PND 85)

In the acquisition phase, a significant effect of time (day) was found ($F_{(3, 168)}=81.31$, $p<0.001$) with all groups completing the test quicker with subsequent testing days (data not shown). No significant effect of treatment, sex or interaction effect of time and treatment or sex and treatment was found for any of the parameters. On the probe day, a significant effect of treatment was found for entries into the SW quadrant ($F_{(13, 59)}=4.612$, $p<0.01$). A *post-hoc* test showed that for females the 0.625,

1.25 and 2.5 mg/kg MA groups had fewer entries into the SW quadrant compared to the control females (Figure 6.1). No significant effect of treatment was found for time spent in the SW quadrant, distance or velocity swam in the MWM (data not shown). There was no significant effect of sex or interaction effect of sex and treatment for any of the parameters.

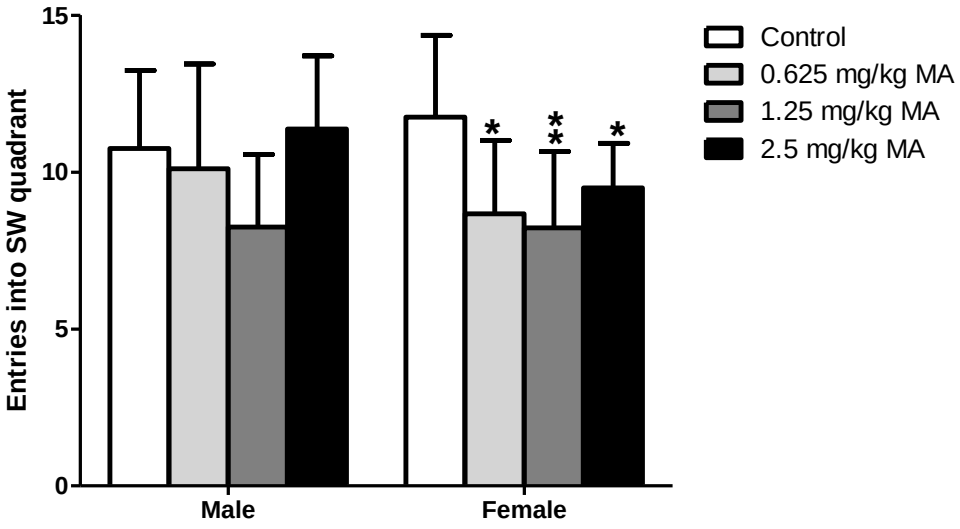

Figure 6.1: Morris water maze. Entries into the SW quadrant on the probe day for males and females on PND 85 (n=16/group). Data are expressed as Mean+SD; $^{**}p<0.01$, $^{*}p<0.05$ vs. relevant control.

Elevated Plus Maze (PND 113)

A significant effect of sex was found for distance (U=322.00, $p<0.01$), velocity (U=320.00, $p<0.01$), OAT (U=356.00, $p<0.01$) and OAE (U=346.00, $p<0.01$) with males moving less, at a slower speed and having lower OAT and OAE compared to females. No significant effect of treatment was found for any of the parameters (data not shown).

Open Field (PND 113)

No significant effect of sex, treatment or interaction effect between sex and treatment was found for any of the parameters (data not shown).

Home Cage Activity and MA challenge (PND 113)

A significant effect of treatment was found for distance at 100 min (K=15.58, $p<0.05$), 140 min (K=17.84, $p<0.05$), 150 min (K=15.88, $p<0.05$) and 160 min (K=18.59, $p<0.05$). A *post-hoc* test showed that the 1.25 mg/kg MA males had a larger distance travelled at 100, 140, 150 and 160 min compared to the control males (Figure 6.2.1). No significant effect of sex was found for distance travelled.

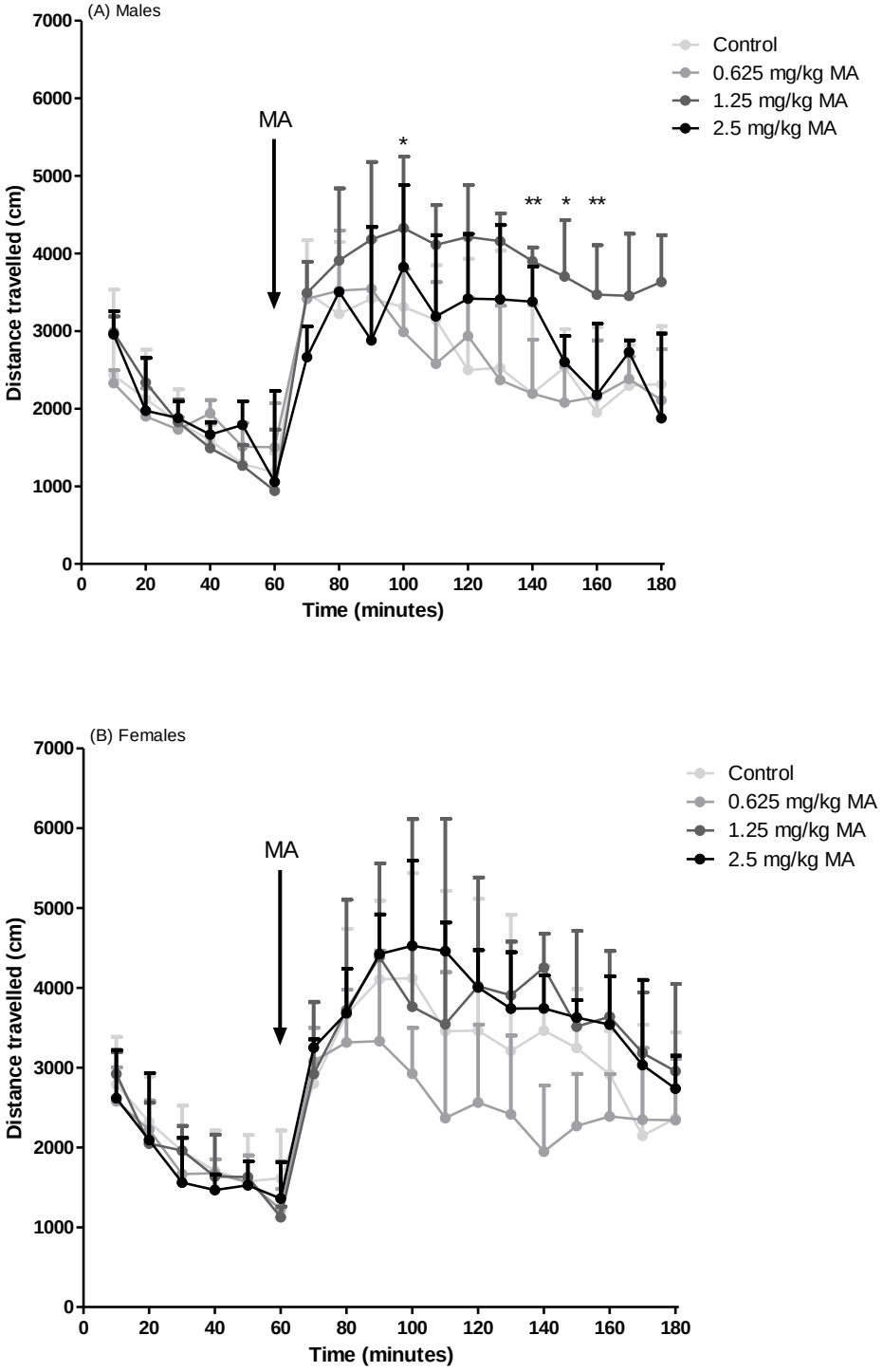

Figure 6.2: Home cage activity. Distance moved in the HCA for (A) males and (B) females on PND 113 (n=15-20/group). Data are expressed as Median and Interquartile range, $^{**}p<0.01$, $^{*}p<0.05$ vs. relevant control.

6.3.2 Experiment 2: Prenatal and Postnatal

Elevated Plus Maze (PND 57)

No significant effect of sex, treatment or interaction effect of sex and treatment was found for any of the parameters (data not shown).

Open Field (PND 57)

No significant effect of sex, treatment or interaction effect of sex and treatment was found for any of the parameters (data not shown).

Forced Swim Test (PND 57)

A significant effect of treatment was found for time spent immobile ($K=11.76$, $p<0.05$). A *post-hoc* test showed that the male MA/VEH group spent more time immobile in the FST compared to controls (Figure 6.3). No significant effect of treatment was found for time spent climbing or swimming in the FST. No significant effect of sex was found for any of the parameters.

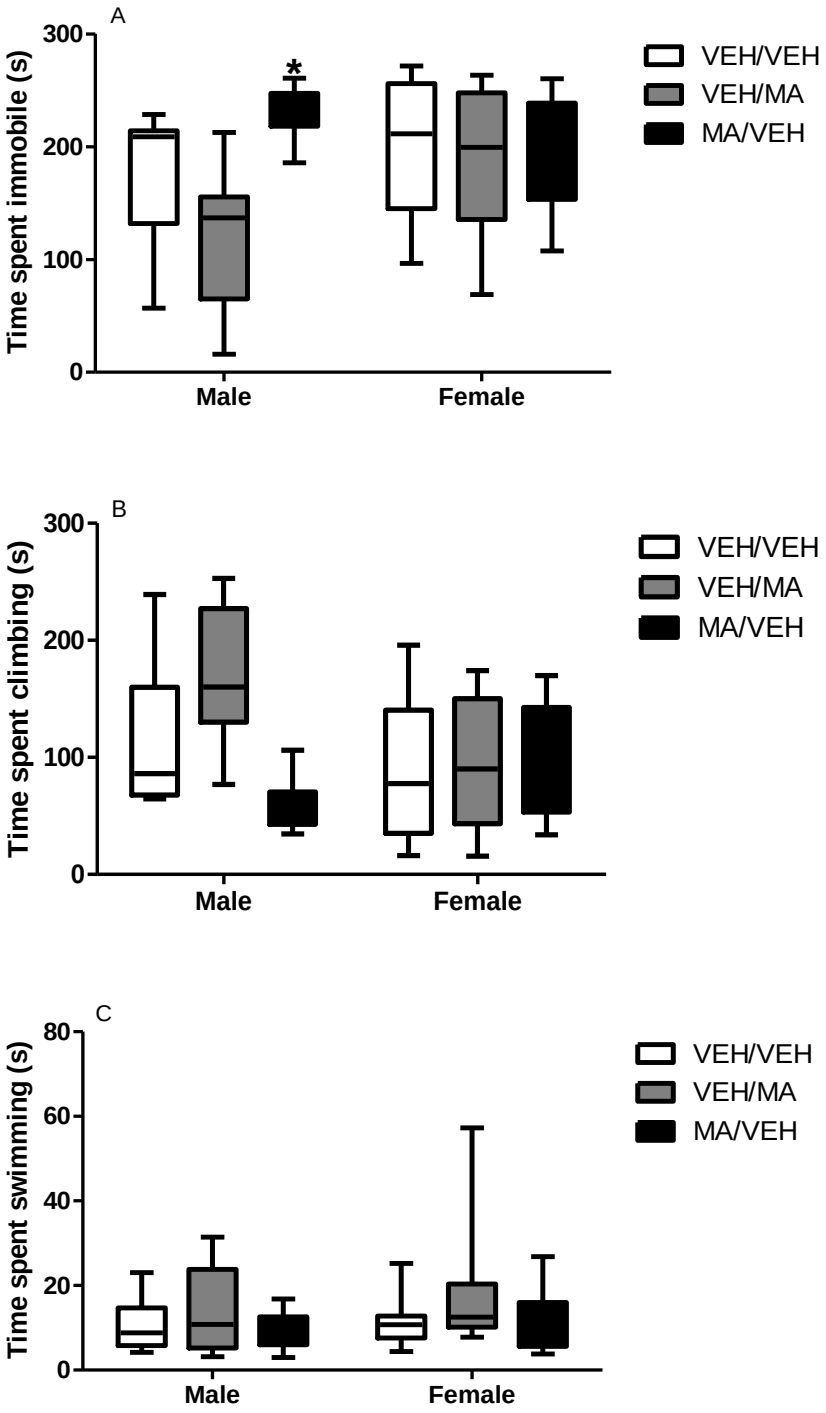

Figure 6.3: Forced swim test. Parameters of the FST including time spent (A) immobile, (B) climbing and (C) swimming for males and females on PND 57 (n=7-10/group). Data are expressed as Median, Interquartile range, min and max; *p<0.05 vs. relevant control.

Morris Water Maze (PND 57)

In the acquisition phase, no significant effect of sex, treatment or interaction effect of sex and treatment was found for any of the parameters (data not shown). On the probe day, no significant effect of sex, treatment or interaction effect of sex and treatment was found for any of the parameters (data not shown).

Home Cage Activity (PND 57)

A significant effect of time was found for distance moved in time bins ($F_{(3.76, 142.99)}$=122.76, p<0.001) in in the HCA with all groups decreasing their distance travelled as time progressed. No significant effect of treatment or interaction effect of time and treatment was found for distance moved in time bins (data not shown).

A significant effect of sex was found for total distance moved ($F_{(1, 38)}$=39.47, p<0.001) in the HCA with females travelling a greater distance than males. A significant interaction effect of sex and treatment was found for total distance moved ($F_{(2, 38)}$=3.65, p<0.05). No significant effect of treatment was found for total distance moved (data not shown).

Elevated Plus Maze (PND 85)

A significant effect of sex was found for distance ($F_{(1, 41)}$=17.37, p<0.001), velocity ($F_{(1, 41)}$=17.06, p<0.001), immobility ($F_{(1, 41)}$=21.10, p<0.001), OAT ($F_{(1, 41)}$=6.10, p<0.05) and OAE ($F_{(1, 41)}$=13.22, p<0.01) with males moving less, at a slower speed and entering and spending less time in the open arms. A significant effect of treatment was found for OAE ($F_{(2, 41)}$=4.19, p<0.05). A *post-hoc* test showed that there was no difference between treatment groups for OAE (data not shown). No significant effect of treatment was found for distance, velocity, immobility or OAT.

A significant interaction effect of sex and treatment was found for OAT ($F_{(2, 41)}$=4.43, p<0.05). No significant interaction effect of sex and treatment was found for distance, velocity, immobility or OAE.

Open Field (PND 85)

A significant effect of sex was found for distance ($F_{(1, 44)}$=7.21, p<0.05), velocity ($F_{(1, 44)}$=7.38, p<0.01) and number of pellets (U=174.50, p<0.01) with males moving less, at a slower speed and also producing less pellets compared to females (data not shown). No significant effect of sex was found for immobility, inner zone duration (IZD) or inner zone entries (IZE). No significant effect of treatment or interaction effect of sex and treatment was found for any of the parameters.

Elevated Plus Maze (PND 113)

A significant effect of sex was found for distance ($F_{(1, 37)}$=6.11, p<0.05) and velocity ($F_{(1, 37)}$=6.16, p<0.05) with males moving less and at a slower speed compared to females. No significant effect of sex was found for immobility, OAT or OAE. A significant effect of treatment was found for distance ($F_{(2, 37)}$=3.65, p<0.05), velocity ($F_{(2, 37)}$=3.65, p<0.05) and immobility ($F_{(2, 37)}$=4.79, p<0.05). A *post-hoc* test showed that there was no difference between treatment groups for distance moved, velocity or immobility (data not shown). No significant effect of treatment was found for OAT or OAE. No significant interaction effect of sex and treatment was found for any of the parameters.

Open Field (PND 113)

A significant effect of sex was found for IZD ($F_{(1, 37)}$=4.35, p<0.05), IZE ($F_{(1, 37)}$=6.36, p<0.05) and number of pellets (U=102.00, p<0.001) with males entering and spending less time in the inner zone and producing more pellets. No significant effect of sex was found for distance, velocity or immobility. A significant effect of treatment was found for IZD ($F_{(2, 37)}$=3.27, p<0.05) and number of pellets (K=18.34, p<0.01). A *post-hoc* test showed that there was no difference between treatment groups for number of pellets or IZD (data not shown). No significant effect of treatment was found for distance, velocity, immobility or IZE. No significant interaction effect of sex and treatment was found for any of the parameters.

6.3.3 Experiment 3: Intermittent Exposure

Elevated Plus Maze (PND 56)

A significant effect of sex was found for distance moved ($F_{(1, 28)}$=11.11, p<0.01), velocity ($F_{(1, 28)}$=11.15, p<0.01), OAT ($F_{(1, 28)}$=9.15, p<0.01) and OAE ($F_{(1, 28)}$=6.45, p<0.05) with males moving less, at a slower speed and entering and spending less time in the open arms. A significant effect of treatment was found for distance moved ($F_{(2, 28)}$=4.11, p<0.05) and velocity ($F_{(2, 28)}$=4.12, p<0.05). A *post-hoc* test showed that there was no significant difference between groups for distance or velocity travelled (data not shown). No significant interaction effect of treatment and sex was found for any other parameters.

Open Field (PND 56)

A significant effect of sex was found for distance moved ($F_{(1, 28)}$=6.95, p<0.05) and velocity ($F_{(1, 28)}$=7.12, p<0.05) in that the males moved less and at a slower speed compared to females. No significant effect of treatment or interaction effect of treatment and sex was found for any of the parameters (data not shown).

Forced Swim Test (PND 56)

No significant effect of sex, treatment or interaction effect of treatment and sex was found for any of the parameters in the FST (data not shown).

6.3.4 Experiment 4: Route of Administration

Elevated Plus Maze and DZP Challenge (PND 56)

A significant effect of sex was found for distance moved ($F_{(1, 30)}$=5.30, p<0.05) and velocity ($F_{(1, 30)}$=5.31, p<0.05) in that the males moved less and at a slower speed compared to females. No significant effect of treatment or interaction effect of sex and treatment was found for any of the parameters in the EPM (Table 6.2).

Drug	Distance moved (cm)	Velocity (cm/s)	Open Arm Time (%)	Open Arm Frequency (%)
Male				
Control	681 ± 206	2.3 ± 0.7	74 ± 24	66 ± 18
MA gavage	986 ± 118	3.3 ± 0.4	51 ± 33	59 ± 15
MA SC	1109 ± 511	3.7 ± 1.7	55 ± 31	58 ± 28
Female				
Control	1192 ± 416	4.0 ± 1.4	75 ± 18	58 ± 9
MA gavage	1341 ± 681	4.5 ± 2.3	74 ± 15	59 ± 15
MA SC	1361 ± 545	4.5 ± 1.8	60 ± 18	53 ± 10

Table 6.2: Elevated plus maze and DZP challenge. Parameters of the EPM for males and females on PND 56 (n=5-7/group). Data are expressed as Mean±SD.

Forced Swim Test and DMI Challenge (PND 56)

A significant effect of treatment group was found for time spent climbing (K=61.35, $p<0.001$) and immobile (K=55.33, $p<0.001$) in the FST preswim. A *post-hoc* test revealed that the MA gavage females spent less time climbing and more time immobile in the FST preswim compared to the control females. DMI also increased time spent immobile and decreased time spent climbing in the FST swim for all groups (Figure 6.4). No significant effect of sex or interaction effect of treatment and sex was found for any of the parameters in the FST.

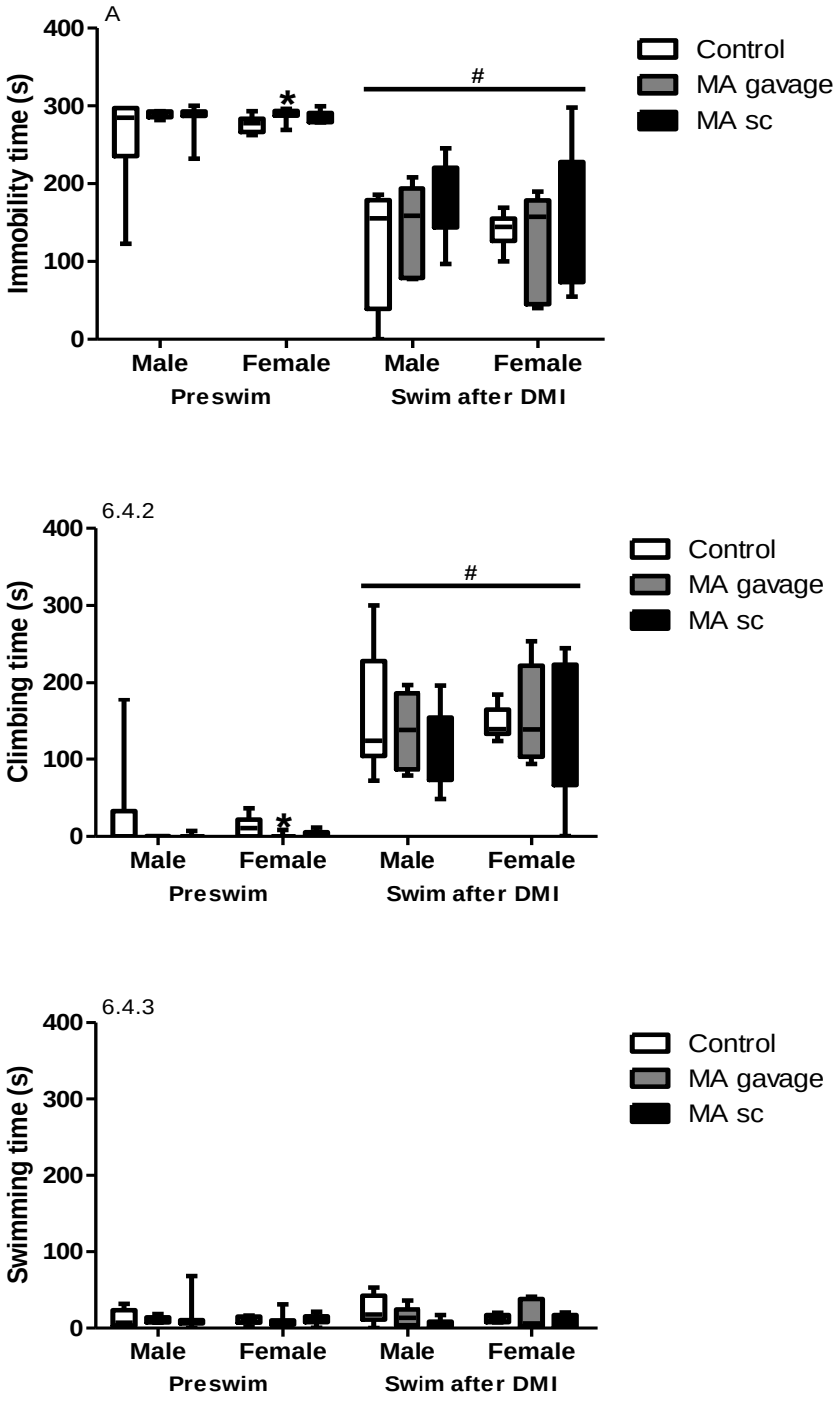

Figure 6.4: Forced swim test. Parameters of the FST with and without DMI challenge including time spent (A) immobile, (B) climbing and (C) swimming for males and females on PND 56 (n=6-7/group). Data are expressed as Median, Interquartile range, min and max; *p<0.05 vs. relevant control, $^{\#}p$<0.05 vs. relevant preswim group.

Home Cage Activity and AMP Challenge (PND 56)

A significant effect of treatment was found for distance moved ($F_{(5, 33)}$=2.57, p<0.05) and velocity ($F_{(5, 33)}$=2.61, p<0.05) in the HCA. A *post-hoc* test revealed that the MA gavage females travelled a greater distance and at a greater speed after 50 min in the HCA compared to the MA sc females (data not shown). A significant effect of time was found for time spent immobile ($F_{(2.78, 91.80)}$=5.44, p<0.01). A *post-hoc* test revealed that the time spent immobile was increased after 20 min and after 60 min in the HCA for all groups. No significant interaction effect of treatment and time was found for any of the parameters in the HCA.

A significant effect of sex was found for total distance moved ($F_{(1, 35)}$=10.66, p<0.01), total immobility time ($F_{(1, 35)}$=10.00, p<0.01) and average velocity ($F_{(1, 35)}$=10.81, p<0.01) in the HCA, in that males moved less and at a slower speed compared to females (data not shown). No significant effect of treatment or interaction effect of treatment and sex was found for any parameters in the HCA.

6.3.5 Summary of Results

	Experiment			
Parameter	**1**	**2**	**3**	**4**
OF	No	No	No	-
EPM	Yes	No	No	-
FST	No	Yes	No	Yes
MWM	Yes	No	-	-
HCA	No	No	-	-
EPM + DZP	-	-	-	No
FST + DMI	-	-	-	No
HCA + AMP/MA	Yes	-	-	No

Table 6.3: Summary table of drug effects found in adult behavioural profiles for experiments 1, 2, 3 and 4. A yes answer means that significant effects of MA were found. An empty space (-) means that this was not performed for this experiment.

	Experiment			
Parameter	**1**	**2**	**3**	**4**
OF	Yes	Yes	Yes	-
EPM	Yes	Yes	Yes	-
FST	No	No	No	No
MWM	Yes	No	-	-
HCA	No	Yes	-	-
EPM + DZP	-	-	-	Yes
FST + DMI	-	-	-	No
HCA + AMP/MA	No	-	-	Yes

Table 6.4: Summary table of sex effects found in adult behavioural profiles for experiments 1, 2, 3 and 4. A yes answer means that significant effects of sex were found. An empty space (-) means that this was not performed for this experiment.

6.4 Discussion

The aim of this study was to determine if MA exposure during pregnancy in rats leads to long-term neurodevelopmental and behavioural alterations in the rat offspring. The present study confirms that prenatal MA exposure at pharmacological doses has adverse effects on the developing offspring and that perturbations in behaviour of the rat offspring are still present in adulthood. The results illustrate that the dose of MA given, the timing of the MA exposure, the frequency of exposure and the route of administration during pregnancy in rats all influence the outcome of the offspring in the adult period. The principal findings for this study show altered anxiety-like behaviour and altered depressive-like behaviour after prenatal exposure to MA.

To determine if prenatal or postnatal MA exposure had any consequence on anxiety-like behaviour in adulthood the EPM was performed in each experiment. The results showed that at the lower doses of prenatal MA (1.25 and 2.5 mg/kg), female offspring exhibited an anxiolytic type behaviour in the EPM in late adulthood (PND 85). MA females were more likely to venture into the open arms more times and also spend longer in the open arms compared to controls. This finding however, was not seen in males, in the other MA doses or at any other time-points in this experiment (experiment 1). This finding was also not found in any of the other experiments. The OF can also be used to assess anxiety-like behaviour and regardless of MA dose, MA exposure times, MA frequencies or MA routes of administration no significant effect was found for this behavioural test. This is in agreement with other studies that reported no differences in baseline behaviour in the EPM or OF for males after prenatal exposure to a higher dose of MA (5 mg/kg sc) when tested in adulthood

(Schutova *et al.*, 2010, Schutova *et al.*, 2009b). Yet, a study by Slamberova *et al.* (2015) showed opposing results in that prenatal MA (5 mg/kg sc) resulted in an anxiogenic effect when tested in the EPM and ultrasound vocalization test in male rats. This was demonstrated as less entries and time spent in the open arms and more entries and time spent in the closed arms compared to controls. This anxiogenic effect of MA was also reported after postnatal MA exposure in male and female rats (Hruba *et al.*, 2012). It is interesting to note that the lower MA doses of 1.25 and 2.5 mg/kg result in an anxiolytic effect, the slightly higher dose of 3.75 mg/kg used in experiments 2-4 had no effect on anxiety-like behaviour and the high dose of 5 mg/kg results in an anxiogenic effect in the EPM. The effects seen may be linked to alterations in the GABA system. A very recent study has reported that sensitization to MA results in upregulation of the mRNAs of transporters (GAT1 and GAT3), ionotropic GABAA receptor subunits (α3 and β1) and the metabotropic GABAB1 receptor (Wearne *et al.*, 2015) and other studies have also looked at the link between GABA and MA abuse (Rubio *et al.*, 2015, Ruda-Kucerova *et al.*, 2015, Godino *et al.*, 2015, Agabio and Colombo, 2015). Unfortunately, this was not evaluated in these studies and so it warrants that future investigations should explore these parameters to uncover the molecular changes behind this long-lasting alteration in anxiety-like behaviour that may possibly be GABA-mediated. A limitation of experiment 4 was the inability to capture baseline behaviour in the EPM before the DZP challenge was administered. Therefore, it is unknown if the MA offspring behaviour in the EPM was comparable to control animals and if DZP did indeed have an anxiolytic effect. When compared to data from the previous experiments control males and females had a baseline %OAT of approximately 30% whereas after DZP administration in experiment 4 this is increased to 75% for both control

males and females. Nonetheless, for the psychopharmacological challenge with DZP in the EPM no significant difference was found for the behavioural response to the drug between groups. This may indicate that the GABAergic system in the offspring is unaffected by prenatal MA exposure.

Locomotor activity in adulthood was also investigated in each experiment to determine if prenatal or postnatal MA exposure had any consequence on exploratory activity in adulthood. To distinguish between anxiety-related behaviour and changes in general locomotor activity behaviour both the OF (novel environment) and the HCA (familiar environment) were examined. For the OF test, no significant effect of MA exposure was found in any of the experiments performed. This has been reported before for baseline activity in the OF after prenatal MA exposure (Schutova *et al.*, 2009b, Schutova *et al.*, 2010) but postnatal MA exposure (5 mg/kg sc) has shown to decrease locomotion and exploratory behaviour in the OF (Hruba *et al.*, 2012). The results for the HCA (experiments 1, 2 and 4) do not highlight any crucial alterations in locomotor activity but in experiment 1 the low dose of 1.25 mg/kg in female rats, increased locomotor activity in the HCA after MA challenge. To the best of our knowledge, there are no other prenatal or postnatal MA studies that have investigated locomotor activity in a familiar environment. Although, MDMA, when given between GD 14 and 20 at a high dose (15 mg/kg sc) has no effect on locomotor activity in the HCA yet, increased activity in the OF for male and female offspring (Thompson *et al.*, 2009b). This study shows that anxiety-related locomotor activity is altered by prenatal MDMA but that general locomotor activity remains unchanged, and vice versa for MA. The laboras test is another measure of locomotor activity used in rodents and this has been previously employed for prenatal MA

studies but this is similar to the OF in that it is a novel environment for the animal. Nonetheless, the laboras test has revealed similar findings to experiment 1 including decreased immobility (Bubenikova-Valesova *et al.*, 2009) and increased locomotion (Slamberova *et al.*, 2012b) in offspring prenatally exposed to MA, albeit at a much higher dose of MA (5 mg/kg) and via the sc route.

In order to determine if prenatal or postnatal MA exposure had any consequence on depressive-like behaviour in adulthood the FST was performed in each experiment. The results showed that at 3.75 mg/kg of MA (when given prenatally via oral gavage), male offspring (experiment 2) and female offspring (experiment 4) exhibited depressive-like behaviour in the FST in early adulthood (PND 56/57). MA males and females spent more time immobile and less time climbing compared to controls. This was not observed in the lower MA doses (0.625 - 2.5 mg/kg). This is a novel finding as depressive-like behaviour in the FST or other behavioural tests has not been previously explored after prenatal or postnatal MA exposure. One study by Williams *et al.* (2003) used the FST as a stressor to illicit a pituitary and adrenal response after neonatal MA exposure (drug delivered directly to the offspring) however performance in the FST was not evaluated. However, when MDMA is given during pregnancy (10 mg/kg sc, GD 13-20) the sucrose preference test (measure of anhedonia) shows a depressive-like behaviour given that rats had a decreased sucrose preference compared to controls (Galineau *et al.*, 2005). Furthermore, the same study investigated changes in dopaminergic and serotonergic functions and found alterations of 5-HT and 5-HIAA cerebral levels at PND 0 and decreased DA and 5-HT levels in adult rats. Therefore, the depressive-like behaviour noted in the present study may also be linked to long-term or permanent changes in

the DA and 5-HT systems. This finding is quite significant as the clinically relevant dose used in the present study (3.75 mg/kg) is much lower to the dose of MDMA used in the aforementioned study (10 mg/kg). In the saccharin preference test and FST after intermittent, high doses of MDMA (15 mg/kg sc, GD 4, 11 and 18) in adult male and female offspring, no significant effect of prenatal MDMA on depressive-like behaviour was found (Adori *et al.*, 2010). This is in agreement with experiment 3 where no significant effect of acute or intermittent MA during pregnancy was observed. This is most likely due to the lower cumulative doses of MA and MDMA.

Clinically the most common report seen in young children exposed *in utero* to MA is altered cognitive performance such as lower long-term spatial memory, lower visual motor integration, lower verbal memory and lower attention (Chang *et al.*, 2004, Diaz *et al.*, 2014). Therefore, to determine if prenatal MA exposure has any consequence on cognition in adulthood, the MWM was performed in experiments 1 and 2. The results showed that at the lower doses of prenatal MA (0.625, 1.25 and 2.5 mg/kg), female offspring exhibited deficits in cognition in the MWM in late adulthood (PND 85). MA females had fewer entries into the SW quadrant on the probe day compared to controls. This finding was not seen in males or in the other MA doses (3.75 mg/kg). Although, when we look at the time spent in the SW quadrant on the probe day there is no difference between treatment groups. Therefore the animals entered the quadrant fewer times but spent the same amount of time within the quadrant. As there was also no difference in the acquisition days (training) this may indicate that there is no true deficit in their learning and memory. This is in agreement with other preclinical literature that has shown no effect of MA

exposure (5 mg/kg sc) on learning and memory behaviour in the MWM when administered during gestation (Schutova *et al.*, 2008, Hruba *et al.*, 2010, Macuchova *et al.*, 2013, Schutova *et al.*, 2009a) or during both gestation and lactation (Hruba *et al.*, 2009a). Deficits in learning and memory have been reported after 0.625, 1.25, 2.5, 5, 10 and 25 mg/kg MA exposure between PND 11 and 20 (Williams *et al.*, 2002, Williams *et al.*, 2003, Williams *et al.*, 2004, Vorhees *et al.*, 2008). However, these studies have employed neonatal dosing where the pups are delivered the drug directly through sc injection. When given through the breastmilk (via the mother) cognitive deficits are still apparent after 5 mg/kg MA exposure from PND 1-21 in male rats (Hruba *et al.*, 2010) but this was not seen in the present study with postnatal dosing (experiment 2).

An unexpected result was the lack of altered response to psychopharmacological challenges in adulthood. No other studies have investigated adulthood challenges to drugs such as DMI and DZP and so there is no comparison for these results. However, many studies have shown that MA or AMP challenge in adulthood after prenatal exposure to MA highlights a sensitisation to the drug (Slamberova *et al.*, 2011b, Yamamotova *et al.*, 2011, Slamberova *et al.*, 2010b, Schutova *et al.*, 2010, Macuchova *et al.*, 2013, Slamberova *et al.*, 2014, Slamberova *et al.*, 2015). Therefore, the lack of effect of AMP or MA challenge in the present study may be due to the different experimental variables such as the lower doses and the oral route administration for MA exposures. The lack of an altered behavioural response to DZP and DMI challenges may suggest that the GABAergic and noradrenergic systems are unaltered by prenatal MA exposure but this warrants further investigation.

Overall, this study has shown that developmental delays observed in the neonatal period after prenatal MA exposure are persistent throughout life and it confirms that alterations in behaviour are still present in adulthood. The main findings from the present study are decreased anxiety-like behaviour and the increased depressive-like behaviour as evidenced in the EPM and FST, respectively. Furthermore, these changes are only observed at low doses of MA and when administered prenatally via oral gavage. Future preclinical studies should aim to investigate changes in the neurotransmitter systems (including DA, GABA and 5-HT) as these may highlight permanent changes in the brain that may be responsible for the behavioural alterations observed. Further time points of testing in late adulthood and inclusion of other behavioural assessments (such as social interaction) may reveal other development delays that were not measured in the present study.

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Chapter 7:

Alterations in oxidative stress after methamphetamine exposure during pregnancy or lactation in rats

Abstract

Although it is not yet fully understood, it is thought that oxidative stress may play a role in MA-induced neurotoxicity. As the developing embryo and foetus are deficient in most antioxidants then they may be at risk of MA-enhanced oxidative stress leading to mitochondrial injury, neuronal apoptosis and nerve terminal degeneration. This in turn may explain the neurodevelopmental deficits seen in these offspring after prenatal MA exposure. However, this is something that has not been investigated clinically or preclinically. Therefore, the aim of this study was to determine the consequences of MA exposure during pregnancy or lactation on oxidative stress in the rat mother and offspring. Pregnant Sprague-Dawley dams received MA (3.75 mg/kg) or control (distilled water) once daily via oral gavage from gestation day 7-21 or postnatal day 1-21. A range of well-recognised oxidative stress parameters were analysed in the dams (PND 21) and offspring (PND 1, 29, 57, 85, 113). Our results showed that parturition and/or lactation increased oxidative stress significantly compared to non-pregnant females however prenatal or postnatal MA had no significant effect in the mothers or offspring. Therefore, the neurodevelopmental delays and behavioural alterations seen both in the neonatal and adult rat, after prenatal MA, may be unrelated to oxidative stress.

7.1 Introduction

The mechanism by which MA induces its neurotoxic effect is not yet fully understood however, emerging evidence has shown a role for oxidative stress in MA-induced neurotoxicity. MA causes increased concentration of DA in the cytosol and this is then subject to increased oxidation to form DA quinine (Wells *et al.*, 2005). DA quinine undergoes redox cycling that can lead to oxidative stress, mitochondrial injury, neuronal apoptosis and nerve terminal degeneration (Schep *et al.*, 2010, Wells *et al.*, 2005, Ares-Santos *et al.*, 2013). Evidence from clinical studies of chronic MA users have shown increased levels of lipid peroxidation (4-HNE and MDA) in the caudate and frontal cortex (Fitzmaurice *et al.*, 2006). Antioxidant levels (CuZnSOD, GSH and uric acid) are also increased in the caudate of MA users which could suggest a compensatory mechanism following MA insult (Mirecki *et al.*, 2004b). Evidence from preclinical studies of rats exposed to acute and repeated doses of MA also shows increased levels of lipid peroxidation (as measured using the TBARS assay) and antioxidant levels (SOD) in the striatum (Açikgöz *et al.*, 1998). Other preclinical studies have also found similar effects (Cadet *et al.*, 1994, Hirata *et al.*, 1995, Jayanthi *et al.*, 1998, Melo *et al.*, 2009, Tokunaga *et al.*, 2008, Thrash-Williams *et al.*, 2013).

The developing embryo and foetus are significantly deficient in most antioxidants *in utero*. In the absence of adequate antioxidative enzymes or pathways for the repair of oxidative DNA damage, the embryo and foetus may be at high risk of MA-enhanced oxidative stress leading to mitochondrial injury, neuronal apoptosis and nerve terminal degeneration. This in turn may explain the neurodevelopmental and behavioural effects found in offspring after exposure to MA during pregnancy

(McDonnell-Dowling *et al.*, 2014, McDonnell-Dowling and Kelly, 2015a, Chaikind and Corman, 1991, Smith *et al.*, 2006, Chang *et al.*, 2004). However, this is something that has not been investigated clinically and the preclinical literature relating to this is lacking. Of the preclinical literature that exists, MA administration during pregnancy at 20 or 40 mg/kg (GD 14 or 17) results in increased DNA oxidation in the foetal mouse brain (Jeng *et al.*, 2005, Wong *et al.*, 2008) and at 5 mg/kg (GD 8 to 22) results in increased MDA in the neonatal retina of the eye on PND 21 (Melo *et al.*, 2005). To the best of our knowledge, this is the only evidence that exists for MA-induced oxidative stress after prenatal insult. Although these studies show important findings, the experimental protocols are not clinically relevant and so it is still unknown if these effects are related to the neurodevelopmental and behavioural effects found clinically. Using a clinically relevant model of MA exposure in pregnancy with lower doses of MA may therefore yield different results. Thus, the aim of this study was to investigate the alterations in oxidative stress after MA exposure during pregnancy or lactation in rats. In order to do this a number of experiments were performed. These experiments examined the effects on oxidative stress of MA alone in adult females, pregnancy alone in adult females, MA alone in pregnant females and finally the effects of MA exposure during pregnancy or lactation in the resultant offspring.

7.2 Materials and Methods

Experiment 1

The aim of experiment 1 was to investigate adult male and female oxidative stress in the striatum and frontal cortex after chronic exposure to MA.

Experiment 2

The aim of experiment 2 was to investigate maternal oxidative stress in the striatum and frontal cortex of the dams after exposure to MA during pregnancy or lactation.

Experiment 3

The aim of experiment 3 was to investigate the effects of pregnancy alone on oxidative stress in the striatum and frontal cortex of the dams.

Experiment 4

The aim of experiment 4 was to investigate the effects of MA alone on oxidative stress in the striatum and frontal cortex of non-pregnant females.

Experiment 5

The aim of experiment 5 was to investigate offspring oxidative stress in the striatum of the offspring after exposure to MA during pregnancy or lactation.

7.2.1 Animal Housing

Adult male (275–325 g, approx. 4 months old) and female (275–325 g, approx. 4 months old) Sprague-Dawley rats were used for this study. All females were bred in-house; all males came from Charles River (Kent, U.K.) and animals were habituated for one week from arrival. After mating, all female rats were housed singly in plastic bottom cages with appropriate bedding material (Gold flake sawdust, Harlan, UK) and additional nesting materials (unbleached cotton and Nesteldown bedding, Petworld, Galway). All animals were maintained under standard laboratory conditions under artificial 12-h light/dark cycle (lights on from 08:00h) and temperature was maintained at 20-24^{o}C with relative humidity at 35-60%. Food and water were provided *ad libitum*. Following littering, the rat pups remained with their biological dams until PND 21, at which point the pups were weaned. Cross-fostering

was not employed in this study in order to mimic clinical scenarios but also to ensure that active drugs present in the MA mother are not passed onto the control pups via breastmilk or urinary and faecal excretions (McDonnell-Dowling and Kelly, 2015c). The pups stayed with their siblings for 7 days until PND 28, at which point they were then separated by sex; littermates of the same sex remained together throughout adulthood. All experiments were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway (12/NOV/07) and in compliance with the European Communities Council directive 86/609.

7.2.2 Mating

There was a male:female ratio of 1:3 for mating. The animals were left undisturbed and allowed to habituate for one week before the study began. Female rats (3) were housed overnight with one sexually mature male rat ensuring that the same females remained together where possible. At the beginning of the light phase the following morning, vaginal smears were taken from all females to check for the presence of sperm. All smears were examined under a light microscope. GD 0 was deemed to be the day that sperm was present in the smear, at which point the female was then removed and singly housed.

7.2.3 Gestation Period and Deliveries

The females were checked daily prior to the start of the dosing period (GD 7-PND21) and the females were weighed daily. The expected day of delivery (birth) is GD 21-22 (Daston *et al.*, 2004). Deliveries that occurred before 17.00h were considered to have their and PND 1 on this day and deliveries that occurred after 17.00h were considered to have their PND 1 on the following day.

7.2.4 Drug Treatment

Methamphetamine HCL was purchased from Sigma Aldrich (St. Louis, MO; M8750). Rats were assigned randomly to control or MA-treated groups based on body weights and likelihood of pregnancy. The dose of MA given was 3.75 mg/kg (unless stated otherwise) at a volume of 1 ml/kg and controls received the VEH alone i.e. 1 ml/kg dH_2O. Our previous dose response study (McDonnell-Dowling *et al.*, 2014) aided in deciding this dose as it compares to a clinically relevant dose in the human scenario and the use of an allometric scale takes into account the body weight and body surface area of a pregnant human versus a pregnant rat (Reagan-Shaw *et al.*, 2008) therefore maintaining the viability of the project and its comparison to clinical situations. Oral gavage was used as this represents the most common route of MA administration in humans, but has before been overlooked in preclinical investigations.

Experiment 1: Adult Male and Female Oxidative Stress

For this experiment 16 females and 16 males were acquired. For MA (5, 10 and 20 mg/kg) or control treatments, the rats were dosed via oral gavage once daily for 14 consecutive days. All rats were sacrificed by decapitation (24 h after last gavage administration). The frontal cortex and striatum were the two brain regions assessed for oxidative stress.

Experiment 2: Maternal Oxidative Stress

For this experiment 52 females and 12 males were acquired. For MA or control treatments, the mothers were dosed via oral gavage once daily at 14.00h until PND 21 (time of weaning). During the gestation period two groups of dams received VEH and one group received MA. During the postnatal period, one group of dams receiving VEH continued to take VEH (VEH/VEH group) and the other was

switched to MA (VEH/MA group), the group of dams receiving MA was switched to VEH (MA/VEH group). One group of mothers received no treatment (NT). This gave four treatment groups. All mothers were sacrificed by decapitation on PND 21 after weaning had occurred (within 1 h of last gavage administration). The frontal cortex and striatum were the two brain regions assessed for oxidative stress.

Experiment 3: Pregnant vs. Non-Pregnant Oxidative Stress

For this experiment 13 females and 12 males were acquired. Non-pregnant females from this study were included and were dosed in the same way to VEH pregnant females. The frontal cortex and striatum were the two brain regions assessed for oxidative stress

Experiment 4: Non-Pregnant Oxidative Stress

For this experiment 13 females were acquired. Non-pregnant females from this study were included and were dosed in the same way to VEH and MA pregnant females to give non-pregnant VEH/VEH and MA/VEH groups. The frontal cortex and striatum were the two brain regions assessed for oxidative stress.

Experiment 5: Offspring Oxidative Stress

For this experiment 52 females and 12 males were acquired. Offspring from experiment 2 were sacrificed on PND 1, 29, 56, 85 and 113. The striatum was the brain region assessed for oxidative stress except for PND 1 when the brain was dissected in half.

7.2.5 Sample Preparation

Brains were removed and immediately stored at −80°C. Prior to analysis, brains were dissected. Brain regions were sonicated with cold phosphate buffered saline (PBS; Sigma-Aldrich, Ireland: P4417) at a volume of 100 mg tissue/ml PBS and

centrifuged at 14000g at 4ºC for 15 min. Resultant supernatants were collected and stored at -80^{o} until use. At the time of analysis, supernatants were thawed on crushed ice and diluted accordingly with PBS.

7.2.6 Bradford Assay

Protein concentrations of all samples was determined using the Bradford assay. Dilutions of bovine serum albumin (BSA; Sigma-Aldrich, Ireland: A8022) were used as standards to create a standard curve with concentrations ranging from 0 to 800 µg/ml. Samples were diluted to 1:20 using PBS (0.01 M). Into a 96 well plate, 20 µl of standard or sample were pipetted in triplicate using reverse pipetting. Bradford Reagent (Sigma-Aldrich Ireland: B6916) was added (230 µl) to all wells and the plate was allowed to incubate at room temperature for 30 min. Protein concentrations were determined at 595nm using a plate reader. Protein concentrations of samples were determined using the standard curve. Results are expressed as µg/ml.

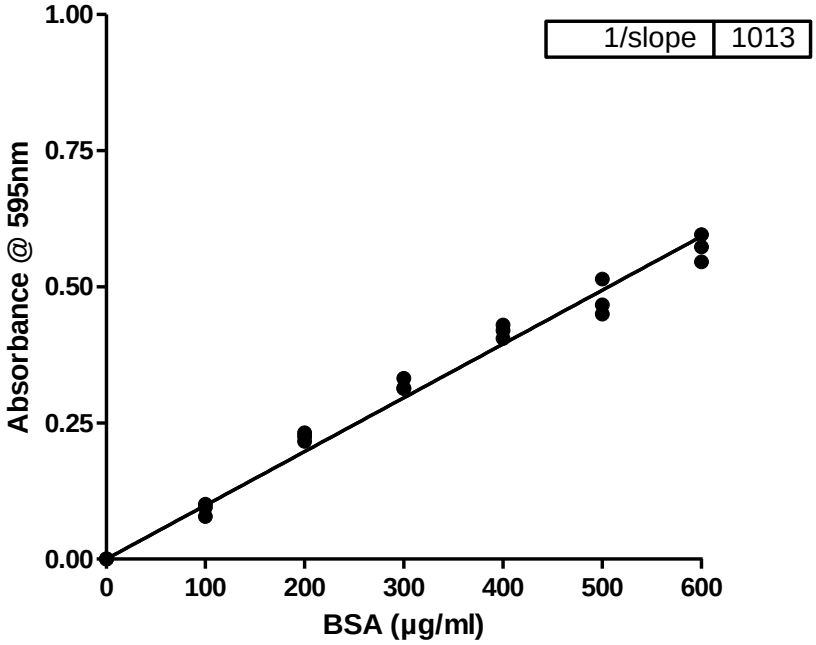

Figure 7.1: Protein standard curve. Data expressed as each replicate (triplicate).

7.2.7 Glutathione Reductase Assay

Glutathione reductase was determined using a commercially available kit (Sigma-Aldrich, Ireland: GRSA). The cuvette assay was converted into a 96 well plate assay and optimised. PBS (0.01 M) was used to prepare the following solutions: GR enzyme (1 ml to 1 vial; >1 unit/ml; standard), nicotinamide adenine dinucleotide phosphate (NADPH, 14.8 mg in 8 ml; 0.0022 M), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, 42.84 mg in 36 ml; 0.003 M) and GSSG (100 mg in 70.42 ml; 0.002 M) and all solutions were made and stored at -20^{o}C. Samples (supernatants) were diluted in PBS to 1:5. At the time of analysis all solutions were warmed to room temperature and GSSG was warmed to 26^{o}C. Dilutions of GR were used as standards to create the standard curve with concentrations ranging from 0 to 40 unit/ml. Reagents were combined in the following order: 500µl GSSG, 90 µl PBS, 10 µl of standard (GR) or sample, 250 µl DNTB and 50 µl NADPH. Into a 96 well plate, 200 µl of each

standard or sample was pipetted in triplicate. The rate of absorbance decrease of NADPH at 412nm was used as an indicator for GR activity and concentrations of samples were determined using the standard curve. Results are expressed as GR activity (units/ug protein).

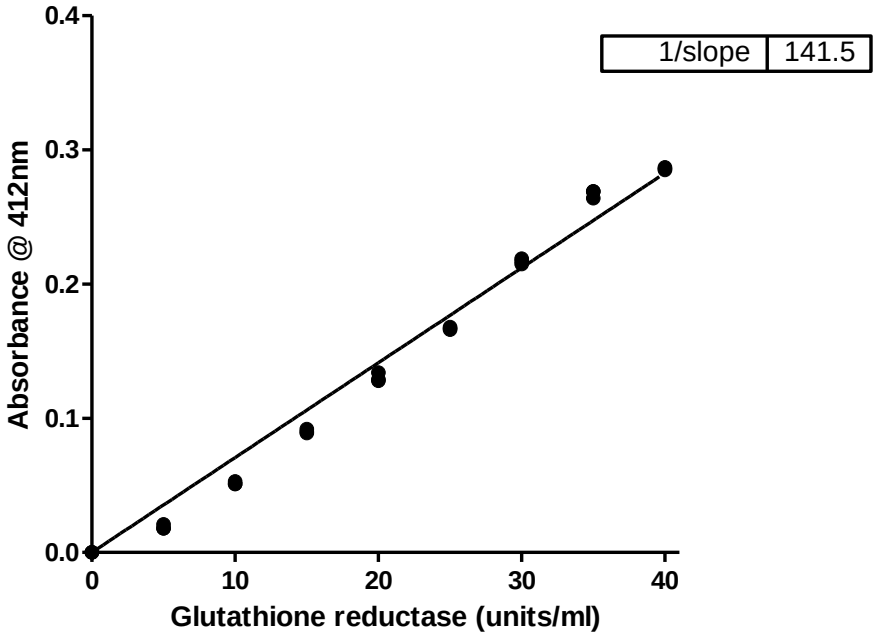

Figure 7.2: Glutathione reductase standard curve. Data expressed as each replicate (triplicate).

7.2.8 Glutathione Peroxidase Assay

Glutathione peroxidase was measured using a commercially available kit (Abcam, U.K.: ab102530). The cuvette assay was converted into a 96 well plate assay and optimised. Assay buffer was used to reconstitute the following solutions: GR (0.22 ml in 1 vial), GSH (0.22 ml in 1 vial), cumene hydroperoxide (CH, 1.25 ml in 1 vial) and GPx (100 µl in 1 vial). NADPH was reconstituted with dH_2O (0.5 ml in 1 vial; 40 mM). NADPH was diluted further with dH_2O (25 µl in 975 µl; 1 mM). All

solutions were made and stored at 4°C. At the time of analysis, assay buffer was warmed to room temperature. Dilutions of NADPH were used as standards to create the standard curve with concentrations ranging from 0 to 100 nmol/well. The rate of absorbance decrease of NADPH at 340nm was used as an indicator for GPx activity.

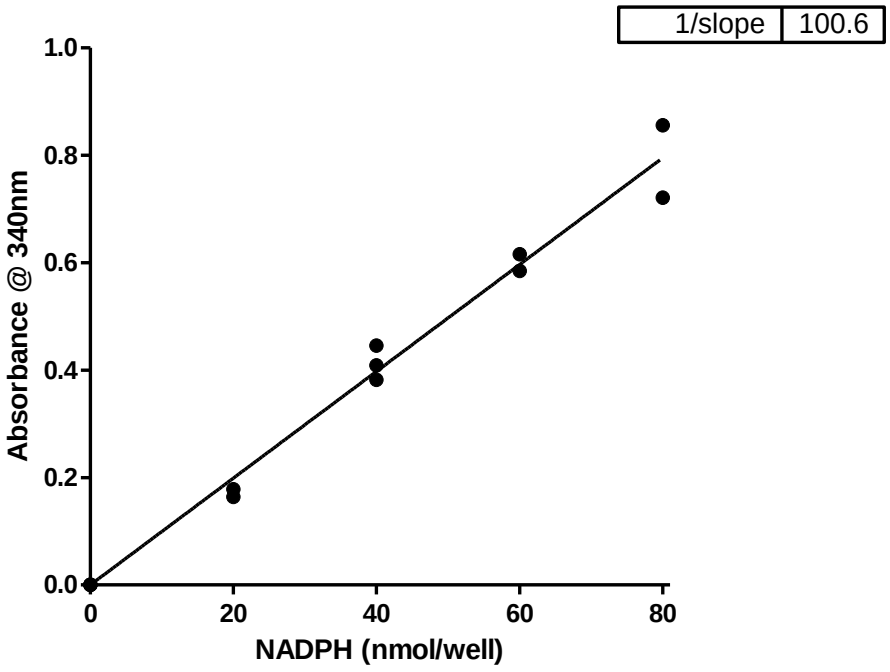

Figure 7.3: Glutathione peroxidase standard curve. Data expressed as each replicate (triplicate).

Reagents were combined to form the reaction mix in the following order: 33 µl assay buffer, 3 µl NADPH, 2 µl GR and 2 µl GSH. Into a 96 well plate, 30 µl of each sample was pipetted in triplicate and for positive control 10 µl GPx was added in triplicate. All wells were brought to a volume of 50 µl using assay buffer and then 40 µl of reaction mix was added. The plate was left on a shaker for 30 sec and then incubated for 15 min. To each well 10 µl of CH was added and the plate was read at 340nm using a plate reader. The plate was then allowed to incubate for 5 min at 25°C

and then read again at 340nm. The concentrations of the sample were determined using the standard curve. Results are expressed as GPx activity (mU/ml/ug protein).

NADPH concentration of samples (nmol) =

[(Sample A1 – Sample A2) – (Blank A1 – Blank A2)]

Figure 7.4: Equation for the calculation of NADPH concentration.

GPx Activity (mU/ml)=

(B/((T2 – T1)* V)) * Sample Dilution

B is the NADPH amount that was decreased between T1 and T2 (in nmol)

T1 is the time of first reading (A1) (in min)

T2 is the time of second reading (A2) (in min)

V is the pretreated sample volume added into the reaction well (in ml)

Figure 7.5: Equation for the calculation of GPx activity.

7.2.9 Thiobarbituric Acid Reactive Substances Assay

Formation of TBARS was used as a method for measurement of lipid peroxidation. PBS was used for dilutions (0.01 M phosphate buffer). MDA (Sigma-Aldrich, Ireland: 63287) was dissolved in PBS (3.135 mg in 5ml; 0.02 M). This was further diluted with PBS to give a 0.0004 M solution. Acetic acid solution (AA; Sigma-Aldrich, Ireland: 45731) was diluted using PBS (1:5) and brought to a pH of 3.5. Thiobarbituric acid (TBA; Sigma-Aldrich, Ireland: T5500) was dissolved in AA solution (2120 mg in 200 ml). Sodium dodecyl sulphate solution (SDS; Sigma-Aldrich, Ireland: 05030) was diluted in PBS to give a 10% solution. Samples (supernatants) were diluted in PBS to 1:3. All solutions were made and stored at -

20ºC and at the time of analysis all solutions were warmed to room temperature. Dilutions of MDA were used as standards to create the standard curve with concentrations ranging from 0 to $4x10^{-4}$ M. Reagents were combined in the following order: 200 µl standard or sample, 200 µl SDS and 500 µl TBA. All standards and samples were left on a heating block for 1 h and then centrifuged at 4ºC for 10 min at 3500g. Into a 96 well plate, 200 µl of each standard or sample was pipetted in triplicate and TBARS was determined by the absorbance at 532 nm. Results are expressed as MDA concentrations (M/ug protein).

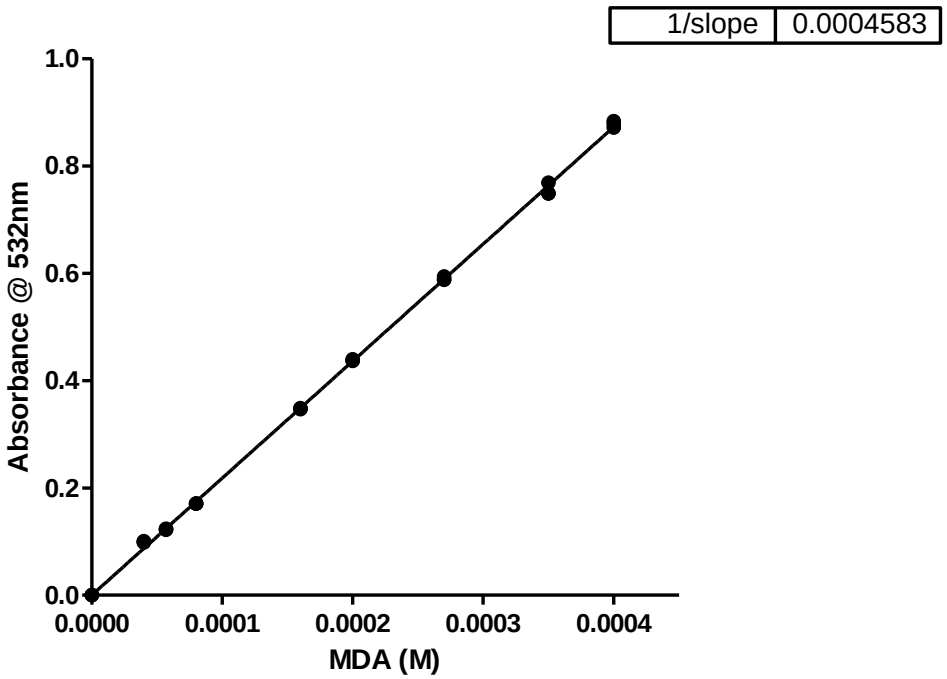

Figure 7.6: Malondialdehyde standard curve for TBARS. Data expressed as each replicate (triplicate).

7.2.10 Superoxide Dismutase Assay

Superoxide dismutase was determined using a commercially available kit (Sigma-Aldrich, Ireland: 19160). WST working solution (WST) was diluted in buffer

solution (1 ml in 19 ml) and enzyme working solution was diluted in buffer solution (15 µl in 2.5 ml). Samples (supernatants) were diluted in PBS (0.01 M phosphate buffer) to 1:50. All solutions were made and stored at 4ºC. All samples and blanks were done in duplicates.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Blank 1	Blank 1	Sample 4	Sample 4	Sample 8	Sample 8	Sample 12	Sample 12	Sample 16	Sample 16	Sample 20	Sample 20
2	Blank 3	Blank 3	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2
3	Sample 1	Sample 1	Sample 5	Sample 5	Sample 9	Sample 9	Sample 13	Sample 13	Sample 17	Sample 17	Sample 21	Sample 21
4	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2
5	Sample 2	Sample 2	Sample 6	Sample 6	Sample 10	Sample 10	Sample 14	Sample 14	Sample 18	Sample 18	Sample 22	Sample 22
6	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2
7	Sample 3	Sample 3	Sample 7	Sample 7	Sample 11	Sample 11	Sample 15	Sample 15	Sample 19	Sample 19	QC	QC
8	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2	Blank 2

Table 7.1: Plate layout for SOD activity assay. Sample arrangement on plate.

20 µl of dH_2O was pipetted into all blank 1 and blank 3 wells and 20 µl of sample was pipetted into sample and blank 2 wells (each sample had its own blank 2). WST (200 µl) was added to each well and dilution buffer (20 µl) was added to all blank 2 and blank 3 wells. Enzyme working solution (20 µl) was pipetted into all sample and blank 1 wells. The plate was allowed to incubate at 37ºC for 10 min and then read at 450nm using a plate reader.

SOD activity (inhibition rate %) =

{[(Ablank 1 - Ablank 3) – (Asample - Ablank 2)]/ (Ablank 1 - Ablank 3)} x 100

Figure 7.7: Equation for the calculation of SOD activity (inhibition rate %).

The rate of the reduction with O2 are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore the inhibition rate of XO activity at

450nm was used as an indicator for SOD activity. Results are expressed as inhibition rate of SOD activity (%inhibition/ug protein).

7.2.11 Statistical Analysis

All figures representing the data from the testing period were constructed using Graphpad Prism 5.0. The data were then analysed using the statistical package SPSS 22.0. Data were firstly assessed to determine if it displayed normality of distribution and homogeneity of variance (Shapiro-Wilks and Levene's test $p>0.05$). For the parametric data tests used included a Two-Way ANOVA or a One-Way ANOVA where appropriate and Student-Newman Keuls *post-hoc* tests were used to define where the significance lay. For the non-parametric data tests used included a Kruskal-Wallis test to compare the effect of treatment groups and Mann-Whitney U tests were used to define where the significance lay. The level of significance was set at $p<0.05$ except when the Bonferroni correction was employed when the level of significance was set at $p<0.008$ (due to multiple comparisons).

7.3. Results

7.3.1 Experiment 1: Adult Male and Female Oxidative Stress

Glutathione Reductase

No significant effect of sex was found for GR concentration therefore male and female groups were combined for statistical analysis. A significant effect of treatment was found for GR concentration in the striatum ($F_{(3, 28)}=2.90$, $p<0.05$) but not in the frontal cortex (Figure 7.8). *A post-hoc* test revealed that the 20 mg/kg group had a lower GR concentration than the 5 mg/kg group.

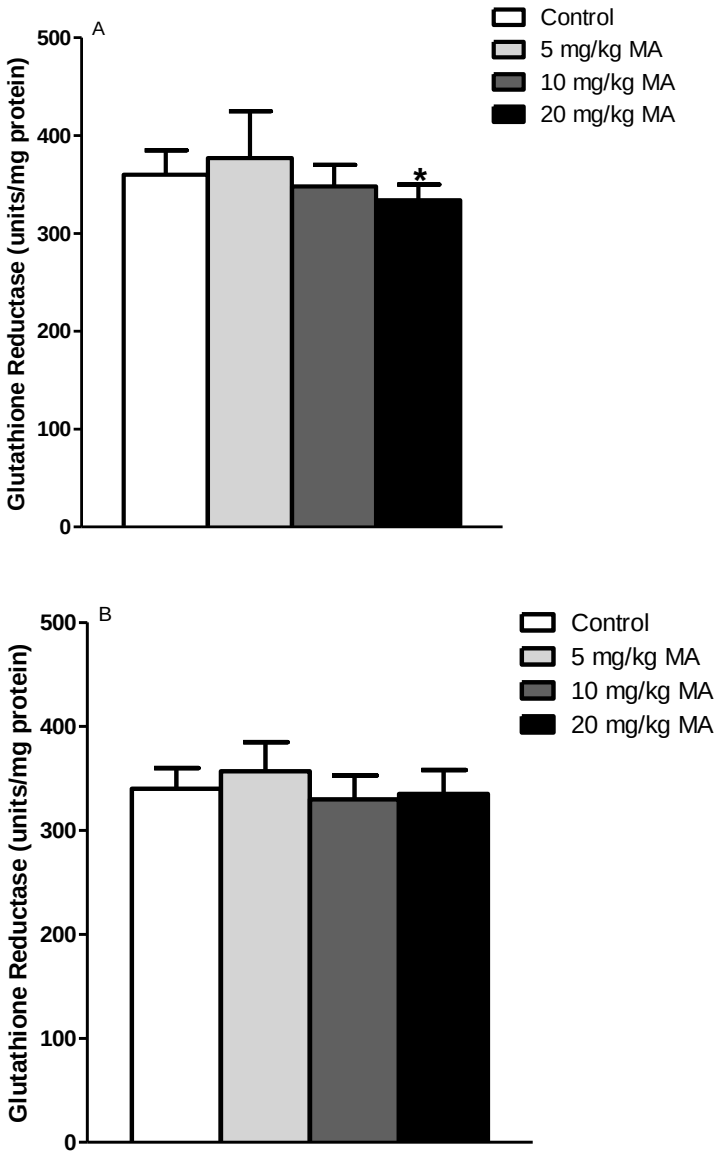

Figure 7.8: Glutathione reductase concentration. GR concentrations in the (A) striatum and (B) frontal cortex of adult rats (n=8/group). Data are expressed as Mean+SD; *p<0.05 vs. 5 mg/kg.

Thiobarbituric Acid Reactive Substances

No significant effect of sex was found for MDA therefore male and female groups were combined for statistical analysis (Figure 7.9). No significant effect of treatment was found for MDA however, there was a strong trend for the 20 mg/kg MA group to decrease MDA concentration in the striatum compared to the control.

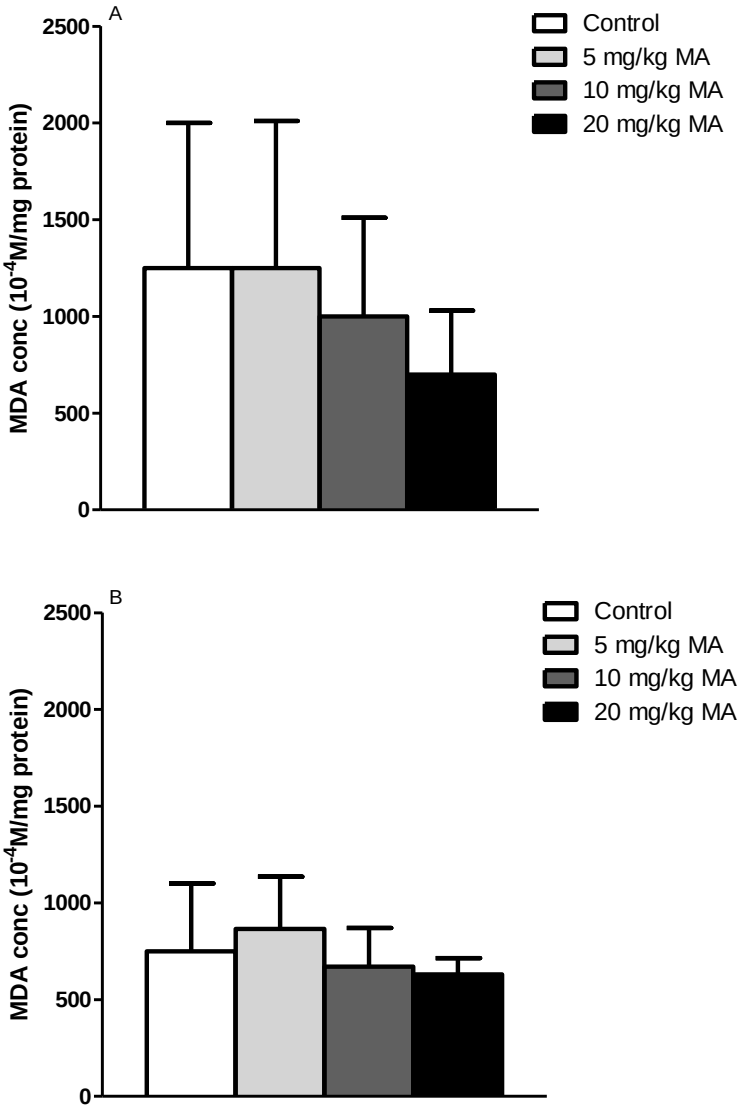

Figure 7.9: TBARS concentration. TBARS concentrations in the (A) striatum and (B) frontal cortex of adult rats (n=8/group). Data are expressed as Mean+SD.

7.3.2 Experiment 2: Maternal Oxidative Stress

Protein

No significant effect of treatment was found for protein concentration in the striatum or frontal cortex (data not shown).

Glutathione Reductase

No significant effect of treatment was found for GR concentration in the striatum or frontal cortex (data not shown).

Thiobarbituric Acid Reactive Substances

No significant effect of treatment was found for MDA concentration in the striatum or frontal cortex (data not shown).

Superoxide Dismutase

No significant effect of treatment was found for SOD activity in the striatum or frontal cortex (data not shown).

Glutathione Peroxidase

No significant effect of treatment was found for GPx activity in the striatum or frontal cortex (data not shown).

7.3.3 Experiment 3: Pregnant vs. Non-Pregnant Oxidative Stress

Protein

A significant effect of treatment was found for protein concentration in the striatum ($U=2.00$, $p<0.05$) and frontal cortex ($U=3.00$, $p<0.05$) (Figure 7.10). Pregnant females had a lower protein concentration than non-pregnant females in the striatum and frontal cortex.

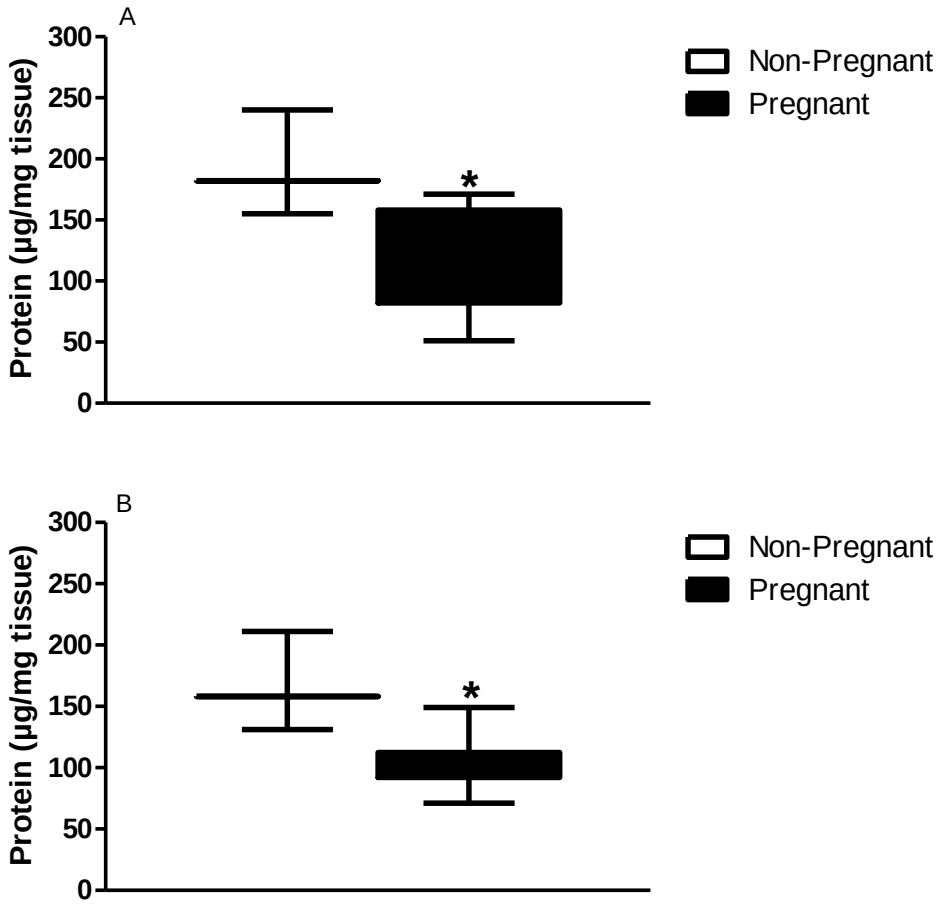

Figure 7.10: Protein concentration. Protein concentration per weight of tissue in the (A) striatum and (B) frontal cortex for pregnant (n=10) and non-pregnant females (n=3). Data are expressed as Median, Interquartile range, min and max; *p<0.05 vs. non-pregnant.

Glutathione Reductase

A significant effect of treatment was found for GR concentration in the striatum (U=0.00, $p<0.01$) and frontal cortex (U=0.00, $p<0.05$) (Figure 7.11). Pregnant females had a higher GR concentration than non-pregnant females in the striatum and frontal cortex.

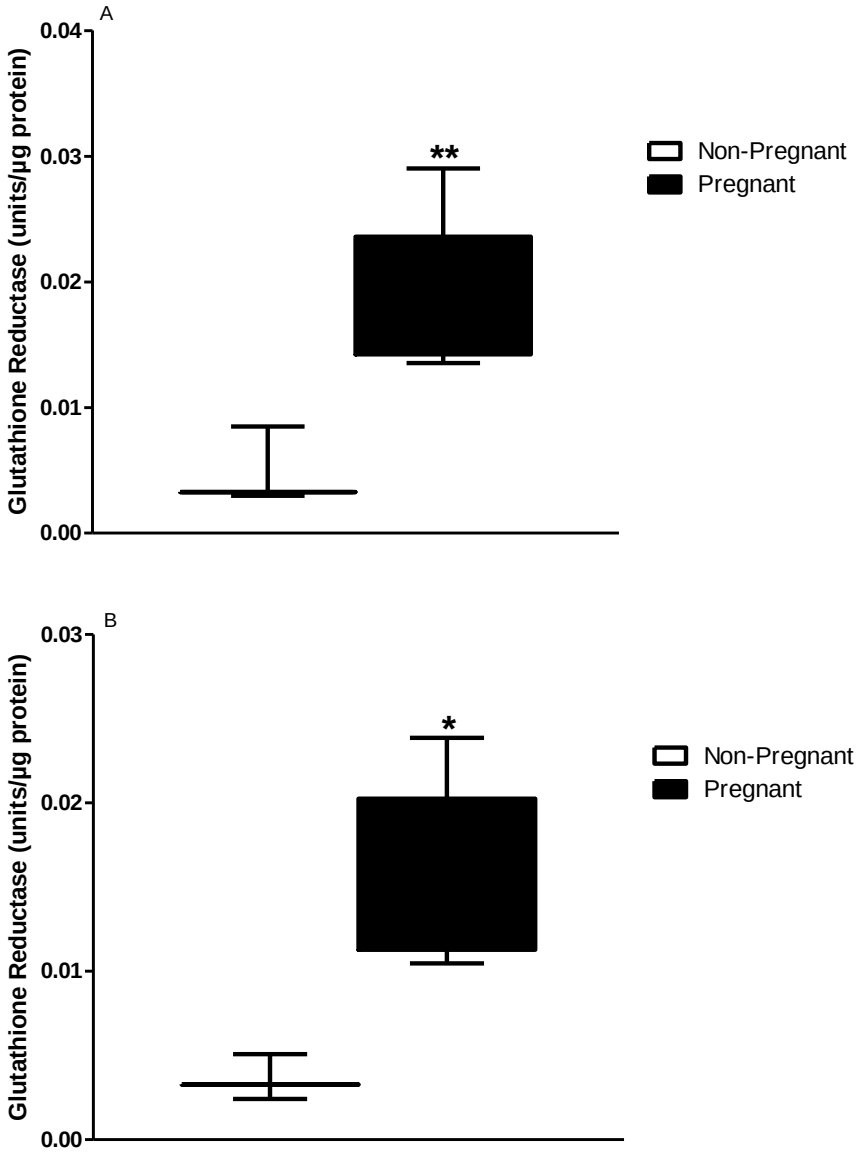

Figure 7.11: Glutathione reductase concentration. GR concentrations in the (A) striatum and (B) frontal cortex of pregnant (n=10) and non-pregnant females (n=3). Data are expressed as Median, Interquartile range, min and max; **$p<0.01$, *$p<0.05$ vs. non-pregnant.

Superoxide Dismutase

A significant effect of treatment was found for SOD activity in the striatum (U=0.00, $p<0.01$) and frontal cortex (U=0.00, $p<0.01$) (Figure 7.12). Pregnant females had lower SOD activity than non-pregnant females in the striatum and frontal cortex.

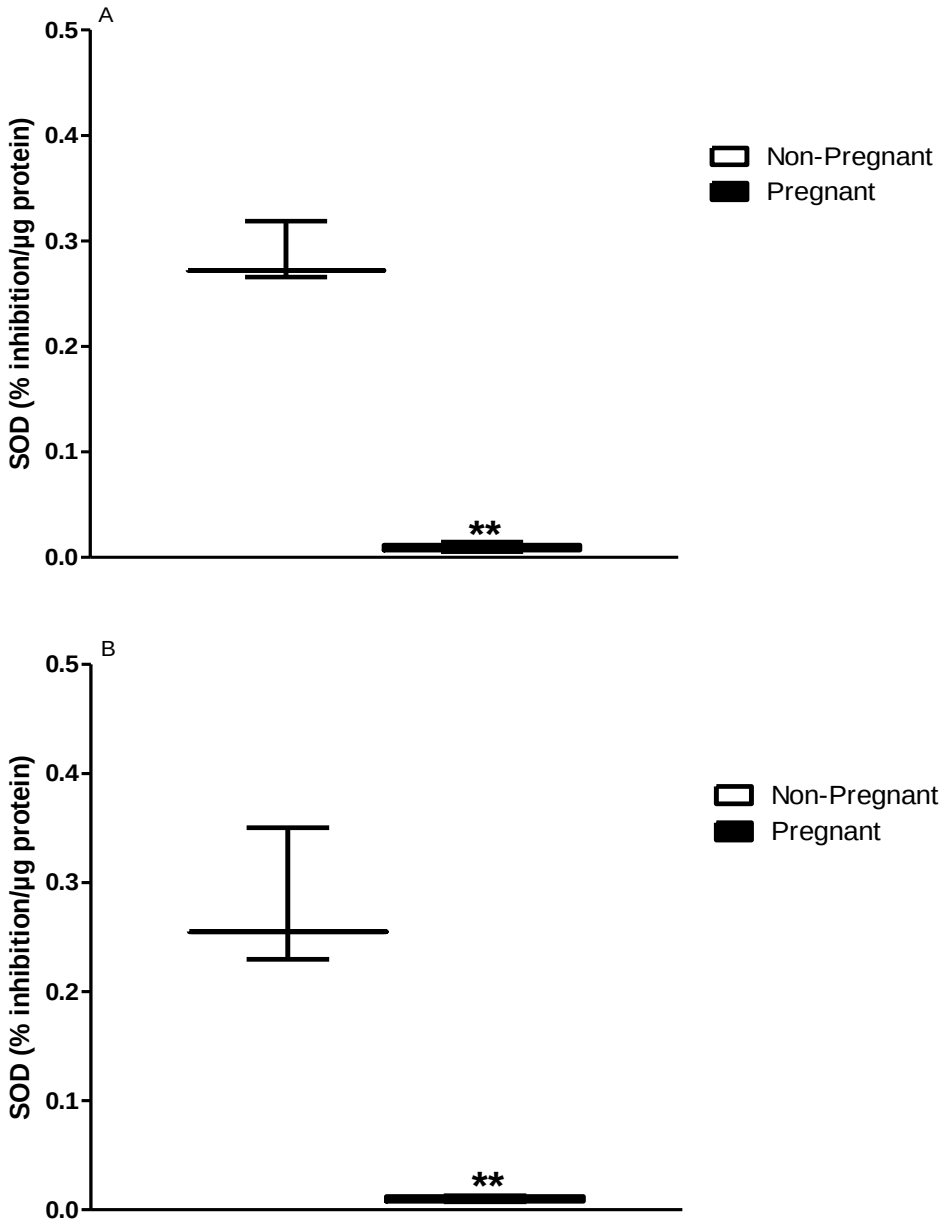

Figure 7.12: SOD activity. SOD activity in the (A) striatum and (B) frontal cortex for pregnant (n=10) and non-pregnant females (n=3). Data are expressed as Median, Interquartile range, min and max; **$p<0.01$ vs. non-pregnant.

7.3.4 Experiment 4: Non-Pregnant Oxidative Stress

Protein

No significant effect of treatment was found for protein concentration in the striatum or frontal cortex (data not shown).

Glutathione Reductase

No significant effect of treatment was found for GR concentration in the striatum or frontal cortex (data not shown).

Superoxide Dismutase

No significant effect of treatment was found for SOD activity in the striatum or frontal cortex (data not shown).

7.3.5 Experiment 5: Offspring Oxidative Stress

Protein

A significant effect of treatment on PND 1 was found ($F_{(2, 28)}$=6.61, p<0.01). *A post-hoc* test revealed that the MA/VEH females had a higher protein concentration than controls (Figure 7.13). A significant effect of sex was found for protein concentration on PND 57 ($F_{(1, 30)}$=6.10, p<0.05) in that females had a lower protein concentration than males. No significant effect of sex was found for PND 1, 29, 85 or 113. No significant effect of treatment was found for PND 29, 57, 85 or 113. No significant interaction effect of treatment and sex was found for PND 1, 29, 57, 85 or 113. A significant effect of time was found ($F_{(2.95, 82.66)}$=42.61, p<0.001) in that PND 29, 57, 85 and 113 samples all had a higher protein content than PND 1 samples and PND 29 was shown to have the highest protein content of all.

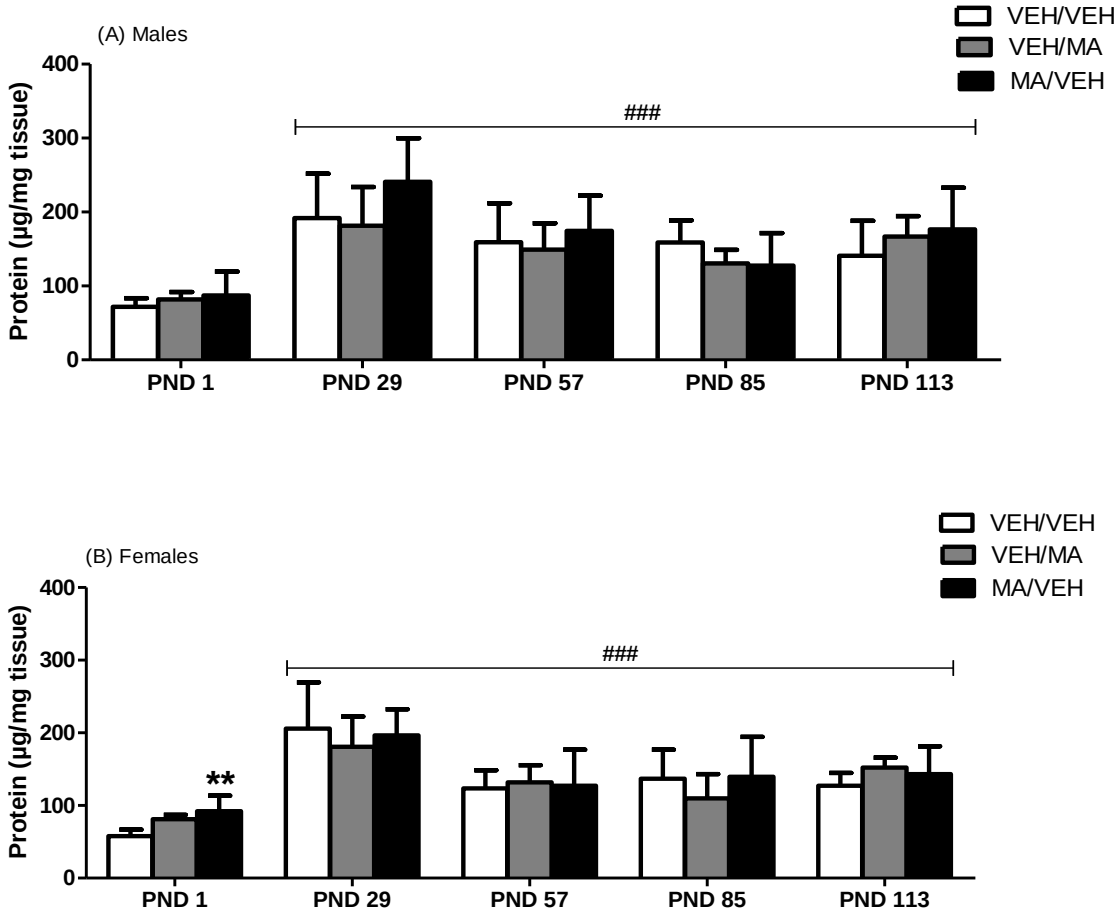

Figure 7.13: Protein concentration. Protein concentration per weight of tissue in the striatum for (A) male and (B) female offspring on PND 1, 29, 57, 85, and 113 (n=5-6/group). Data are expressed as Mean+SD; $^{###}p<0.001$ vs. PND 1, $^{**}p<0.01$ vs. relevant control.

Glutathione Reductase

A significant interaction effect of treatment and sex was found on PND 85 ($F_{(2, 29)}=4.09$, $p<0.05$) for GR concentration. A significant effect of sex was found for GR concentration on PND 1 ($F_{(1, 28)}=15.25$, $p<0.01$), PND 29 ($F_{(1, 30)}=41.89$, $p<0.001$) and PND 85 ($F_{(1, 29)}=9.17$, $p<0.01$) in that females had a higher GR concentration than males (Figure 7.14). No significant effect of sex was found for PND 57 or 113.

No significant effect of treatment was found on PND 1, 29, 57, 85 or 113. No significant interaction effect of treatment and sex was found on PND 1, 29, 57 or 113. A significant effect of time was found ($F_{(3.02, 81.46)}$=36.34, p<0.001) in that PND 29, 57, 85 and 113 samples all had a higher GR concentration than PND 1 samples and in males PND 57 was shown to have the highest GR concentration of all whereas in females this was PND 29.

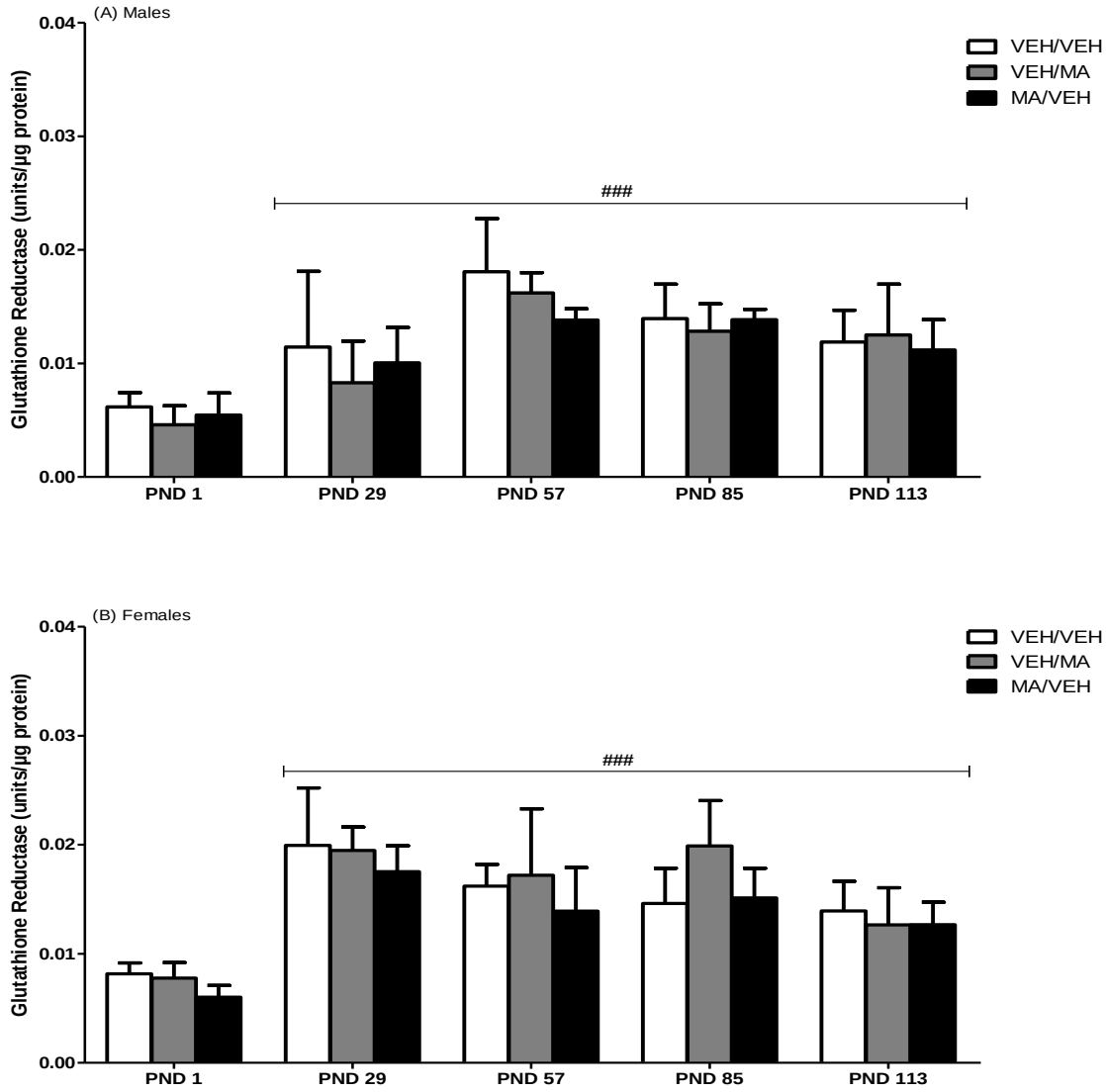

Figure 7.14: Glutathione reductase concentration. Glutathione Reductase concentrations in (A) male and (B) female offspring (n=5-6/group) on PND 1, 29, 57, 85 and 113 in the striatum. Data are expressed as Mean+SD; $^{###}p$<0.001 vs. PND 1.

Superoxide Dismutase

A significant effect of treatment was found for SOD activity on PND 85 ($F_{(2, 30)}$=2.85, p<0.05). *A post-hoc* test revealed that the VEH/MA females had higher SOD activity than controls (Figure 7.15). A significant effect interaction effect of treatment and sex was found for SOD activity on PND 85 ($F_{(2, 30)}$=3.39, p<0.05). A significant effect of sex was found for SOD activity on PND 1 ($F_{(1, 27)}$=6.63, p<0.05), PND 29 ($F_{(1, 30)}$=4.73, p<0.05) and PND 113 ($F_{(1, 30)}$=30.93, p<0.001) in that females had higher SOD activity than males. No significant effect of sex was found on PND 57 or 85. No significant effect of treatment or interaction effect of treatment and sex was found on PND 1, 29, 57 or 113. A significant effect of time was found ($F_{(4, 108)}$=92.08, p<0.001) in that SOD concentration increased with age and then decreased at PND 113 for males and females.

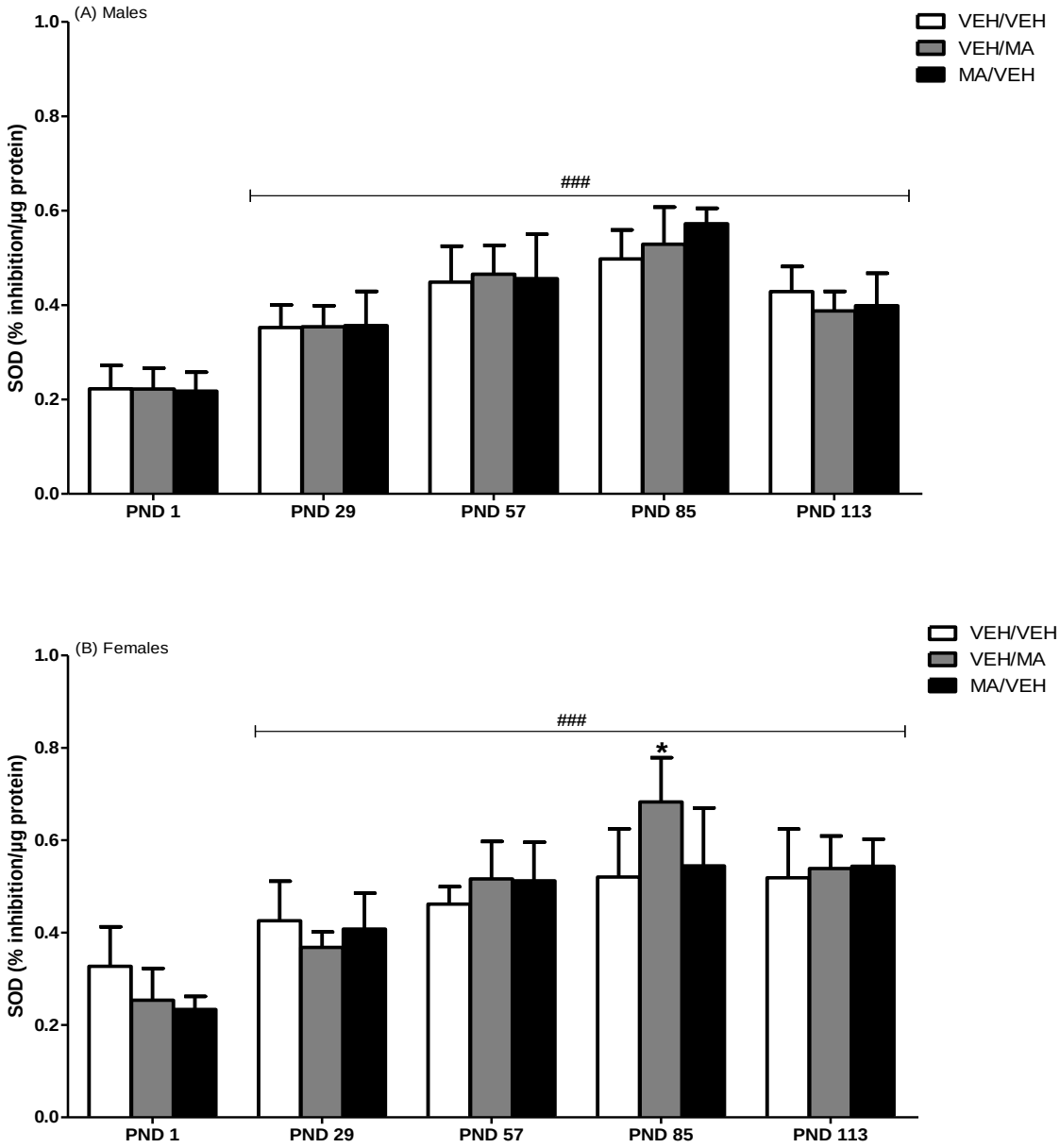

Figure 7.15: SOD activity. SOD activity in the striatum for (A) male and (B) female offspring on PND 1, 29, 57, 85 and 113 (n=5-6/group). Data are expressed as Mean+SD; *p<0.05 vs. relevant control, $^{###}p$<0.001 vs. PND 1.

7.4. Discussion

The aim of this study was to determine if MA exposure during pregnancy or lactation in rats leads to perturbations in oxidative stress. This study investigated the effects on oxidative stress of MA and/or pregnancy and lactation and the effects of

MA exposure during pregnancy or lactation in the resultant offspring. The present study shows that prenatal or postnatal MA exposure at a pharmacological dose has no effect on oxidative stress in the mother or developing offspring. The findings for this study show that pregnancy alone increases oxidative stress but that MA exposure does not alter any oxidative stress parameters.

The first objective of this study was to investigate maternal oxidative stress after exposure to MA during pregnancy or lactation. The results showed that maternal oxidative stress was unaffected by exposure to MA. Of the parameters assessed (GR, TBARS, SOD and GPx), both the VEH/MA and MA/VEH groups were comparable to the control group for both the striatum and frontal cortex. Of the preclinical literature that exists for prenatal MA exposure and oxidative stress none of the existing studies investigated alterations in oxidative stress in the mothers and focused only on the offspring. Therefore, it is unknown if these results are to be expected. In MA exposure studies in adult rats, this dosing regime and dose (3.75 mg/kg po for 14 or 21 days) has not been employed however, a lower dose of MA when administered chronically (2.5 mg/kg ip for 6 days) resulted in increased GSH, Glrx and GR in the STR, decreased GCS in the striatum (STR) and frontal cortex (FC) and decreased GR in the thalamus (Pang *et al.*, 2013). A higher dose of MA when administered acutely (5 mg/kg ip) had no consequence on oxidative stress (TBARS, GPx and SOD) in the STR and prefrontal cortex (PFC) (Acikgoz *et al.*, 2000). The discrepancies in these results are noteworthy however it is difficult to compare these effects when such different experimental parameters were used. The variation in results may be due to the different dose, route or dosing frequency employed. Although, another possibility is the important fact that the females in our

study were pregnant. Hence, it might be possible that parturition alone can also alter these oxidative stress parameters. For that reason, the next objective of the study was to investigate the effects of pregnancy alone on oxidative stress. The total protein content in the pregnant females was less than that of the non-pregnant females. This was assessed by Bradford assay and unfortunately does not tell us anything about specific proteins that may be affected. This has not been reported previously and therefore may warrant further investigation. With regards to the other assays performed, the results showed that oxidative stress was significantly altered by pregnancy/lactation when compared to non-pregnant females of the same age. Of the parameters assessed (GR and SOD), the pregnant females showed alterations in oxidative stress compared to non-pregnant females in both the striatum and frontal cortex. The activity of GR was increased while the SOD activity was decreased in both brain regions for pregnant females. The opposing effects seen in these two enzymes is quite common after MA-induced oxidative stress (Koriem *et al.*, 2013, Zhang *et al.*, 2012, Harold *et al.*, 2000, Flora *et al.*, 2002) and the decrease or almost complete inactivation of the scavenger enzymes seen in this study (SOD) is also known to occur when the antioxidant defence system is overloaded (Krasnova and Cadet, 2009, Cadet and Brannock, 1998). This effect of oxidative stress during and after parturition has been reported clinically. Diaz-Castro *et al.* (2015) showed that parturition increases oxidative damage in the mother but that this may not necessarily be reflected in the neonate in that oxidative damage was lower in the umbilical cord artery. Yuksel and Yigit (2015) also found that oxidative stress can change throughout the pregnancy in that NO and GPx were higher and SOD and CAT were lower in the second and third trimesters compared to the first trimester. Preclinically it has also been shown that lactation can significantly

increase oxidative stress in mice. Levels of H_2O_2 and MDA in lactating mice are higher in the brain than that of non-lactating mice and GPx is also decreased in the brain of these mice (Zheng *et al.*, 2015). The changes in oxidative stress found in the present study after pregnancy and lactation (experiment 3) may therefore disguise any fluctuations in oxidative stress that might have occurred due to MA exposure (experiment 2). If a 'plateau' effect has occurred due to pregnancy or lactation then changes in oxidative stress due to MA exposure may not be detectable. In order to be sure of this the effect of MA exposure on oxidative stress was assessed in non-pregnant females. The same experimental protocol was employed as for the pregnant females in experiment 2 but all females were non-pregnant. The results showed that oxidative stress was not significantly altered by MA exposure when compared to controls. Of the parameters assessed (GR and SOD), the MA females showed no variations in oxidative stress compared to control females in either the striatum or frontal cortex. As the same results were seen in the pregnant females it can be concluded that regardless of pregnancy or lactation, MA did not induce variations in oxidative stress in the present study. The lack of effect seen here is most likely due to the dose and exposure regimen employed. Our extensive review of the literature (Chapter 1 II) showed that low doses of MA given acutely (2 mg/kg) or chronically (0.25 mg/kg) yield a greater effect on oxidative stress than higher doses given acutely (15 mg/kg) or chronically (20 mg/kg) but this deviates from the clinically relevant scenario.

The final objective of this study was to investigate offspring oxidative stress after exposure to MA during pregnancy or lactation. The results showed that offspring oxidative stress was unaffected by exposure to MA during pregnancy or lactation. Of

the parameters assessed (GR and SOD), both the VEH/MA and MA/VEH groups were comparable to the control group for both males and females, in the striatum, at all time points. Although the female VEH/MA group appear to have higher SOD activity on PND 85 compared to controls, this seems to be more likely related to the lower protein concentration of the STR than the MA exposure. Of the preclinical literature that is available, it's been shown that MA administration at 20 or 40 mg/kg (GD 14 or 17) resulted in increased DNA oxidation in the foetal mouse brain and no differences were noted between the two MA doses (Jeng *et al.*, 2005, Wong *et al.*, 2008). MA exposure at 5 mg/kg (GD 8 to 22) also results in increased MDA in the neonatal retina of the eye on PND 21 (Melo *et al.*, 2005) but no studies have evaluated oxidative stress in the brain of offspring after birth. In neonatal rats exposed to MA daily from PND 4-10 (5-10 mg/kg sc), TH enzyme levels and synaptophysin levels were decreased in the STR and PFC and pre-treatment with melatonin (2 mg/kg 30 min prior) prevented these MA-induced reductions (Kaewsuk *et al.*, 2009). The authors hypothesise that melatonin may have provided a protective effect against MA probably via its antioxidant properties. This suggests MA-induced oxidative stress in the neonate brain but when given at a lower dose via the breastmilk of the mother, as in the present study, this is not the case. Our results do show an increase in oxidative stress with increasing age from PND 1 to 85/113 which suggests a maturing of the oxidative stress system during the neonatal and adolescent periods and this has been previously reported clinically and preclinically (Aydin *et al.*, 2015, Wang *et al.*, 2015, Schottker *et al.*, 2015, Rathor *et al.*, 2015).

In conclusion, this is the first study to show that prenatal or postnatal MA exposure does not alter oxidative stress in dams or their offspring. Our results indicate that

both parturition and/or lactation and age increase oxidative stress but that MA exposure has no effect of itself. Therefore, the neurodevelopmental delays and behavioural alterations seen both in the neonatal (chapter 3) and adult rat (chapter 6), after prenatal/postnatal MA, may occur via a different cascade of events. Alternatively, we may not have captured the oxidative stress alterations with these particular assay methods and measurements. In order to investigate this further, future studies should aim to look at a broader spectrum of oxidative stress parameters accompanied by an assessment of the neurotransmitter systems. This in turn would give a better picture of oxidative stress in the dam and offspring and may confirm the present findings that oxidative stress is unrelated to MA-induced neurodevelopmental deficits.

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Chapter 8:

Discussion

8.1 Discussion

With the rising number of pregnant females abusing MA, preclinical studies have aimed to elucidate the consequences of such exposure on the offspring. Our thorough review of the literature revealed that extrapolating the results found in preclinical studies to the clinical scenario is fraught with difficulty as many studies have deviated from an animal model that can be considered clinically relevant. Chapter 1 stressed the lack of consistency among preclinical studies with regards to mating, drug dose, exposure frequency, litter standardisation, cross fostering and behavioural testing as well as many other experimental parameters. Chapter 1 also drew attention to similar inconsistencies in other preclinical studies investigating the effects of MA exposure on oxidative stress such as drug dose, time of exposure, exposure frequency, time point of measurements, brain regions and many more. This extensive literature review therefore shaped the design of the studies that make up this thesis.

The work presented in this thesis aimed to determine the outcome in the neonatal, adolescent and adult offspring after exposure to MA prenatally and/or postnatally using a clinically relevant animal model. Any potential animal model should meet a number of criteria including face, construct and predictive validity as well as having translational value and potential. Thus, the goal of our model was to mimic the clinical scenario as closely as possible and encompass as many aspects of this scenario as possible. The following sections will discuss each individual aspect of our model with these criteria in mind and review the general findings in relation to these.

Dose

One of the main objectives of this study was to translate doses that were clinically relevant to doses that could be employed preclinically and this was performed using an allometric scale (Reagan-Shaw *et al.*, 2008). Although this method does not allow for pharmacokinetic-pharmacodynamic translations (Green and Nutt, 2014) it allows us to approximate clinical doses to preclinical doses. Therefore, a dose of 15 or 30 mg [most commonly abused doses in pregnant females (Golub *et al.*, 2005)] translates to a dose of 1.25 and 2.5 mg/kg respectively, which are classed as 'pharmacological doses' (McDonnell-Dowling and Kelly, 2015). Hence, these doses became the foundation for the dose response study performed in chapter 2. Although they were not included in this thesis, 5 and 10 mg/kg of MA were also investigated; however both of these doses resulted in maternal death at birth, meaning that they would not be suitable to use in subsequent studies. Chapter 3 and the subsequent chapters aimed to explore the consequences of a 'high pharmacological dose' of 3.75 mg/kg (McDonnell-Dowling and Kelly, 2015). This increase in dose aimed to compensate for the pharmacokinetic differences between the rat and human.

With regards to maternal effects, MA at the low pharmacological doses had no consequence on the dams, but at the higher dose of 3.75 mg/kg maternal body weight gain was less than that of the controls. This result was observed in all studies employing this dose albeit transient and only persisting for the first few days after dosing began. It was also correlated to reduced food consumption in these few days. These minimal and transient outcomes in the mothers are preferred as severe malnutrition or anorexia may lead to other complications or consequences in the offspring making it hard to distinguish what outcomes are due to the drug treatment alone. Looking next to the neonatal offspring effects, MA exposure was associated

with both somatic and behavioural developmental delays and in similar fashion to the mothers these effects were interrelated with drug dose. Chapter 2 showed that the developmental delays observed in the offspring were dose-dependent and most notable in the highest dose. While some deficits were noted to a lesser degree with the 1.25 mg/kg, deficits in almost all developmental parameters were observed with the 2.5 mg/kg dose. Chapter 3 and 5 confirmed that prenatal MA exposure was associated with similar delays in neurodevelopment in the offspring at the higher pharmacological dose of 3.75 mg/kg. These combined neonatal results show that the quantity (dose) of drug is a key factor in determining the neonatal outcome and the magnitude of retardations observed.

As mentioned previously the pharmacokinetic properties of MA are greatly different in rats compared to humans and this cannot be overlooked (Table 8.1).

	Route	**Dose**	**$T_{1/2}$**
Rat	sc	3 mg/kg	51 ± 3 min
	ip	3 mg/kg	51 ± 2 min
	iv	1 mg/kg	63 ± 14 min
Human	Smoked	22 mg	12 ± 1 h
	iv	30 mg	9 ± 1 h
	Oral	10 mg	9 ± 4 h
	Intranasal	50 mg	11 ± 1 h

Table 8.1: Summary of the half-life of MA in rats and humans using various routes of administration (Gentry *et al*., 2004, Riviere *et al*., 1999, Cook *et al*., 1992, Cook *et al*., 1993, Schepers *et al*., 2003, Cruickshank and Dyer, 2009).

A limitation of the present model is the inability to mimic these pharmacokinetics of MA in humans. A half-life of 1 hour versus 9-12 hours in rats and humans respectively is quite significant and due to this it is unlikely that the plasma concentrations that the offspring are exposed to in our model are representative of the plasma concentrations in the clinical situation. However, in order to reliably model the plasma concentrations of human MA exposure in rats, and therefore the true concentration that the offspring are exposed to, an alternative model would need to be employed. To the best of our knowledge, the only study to successfully approximate human MA plasma concentrations in rats was by Segal and Kuczenski (2006). This was achieved by MA being administered using continuous iv infusions and the animals were exposed to fluctuating levels of the drug throughout the day. Although this model is desirable in that it achieves clinically relevant plasma concentrations, with this application however, we are deviating from the clinical scenario. With the aforementioned model one cannot control the timings, frequencies and route of drug exposure which the present thesis has successfully achieved. Although this is beyond the scope of the present thesis, it would be of great benefit to have future studies which employ this method. This in turn would give us a true understanding of the neonatal outcome when exposed to plasma concentrations that match those seen in humans.

Exposure timings and frequencies

Another objective of this model was to employ dosing patterns that are clinically relevant and to again ensure construct, face and predictive validity. In order to accurately portray the clinical dosing patterns multiple scenarios were employed including several exposure timings (prenatal and/or postnatal) and numerous

exposure frequencies (acute, intermittent or chronic). Similar maternal bodyweight effects were observed in all MA studies however, for the intermittent exposure study these effects may have been related to increased locomotor activity as opposed to anorectic properties as seen with the chronic dosing. As with the dose of drug, the exposure timing and frequency was also shown to influence the neonatal outcome with the postnatal MA exposure having an equally deleterious effect on the somatic and behavioural development of the offspring. Both prenatal and postnatal exposures were linked with developmental deficits yet, when these exposures were combined it showed that chronic exposure during the gestation and lactation periods had the greatest effect of all with almost all developmental parameters being affected. These results show that MA as a result of being able to pass to the unborn or new-born offspring via the placenta and breastmilk can lead to retardations in the neonatal period. Chapter 4 focused on sporadic dosing periods to explore the possibility that reduced frequency of exposure to MA may lead to less severe retardations in the offspring. While employing the same 'high pharmacological dose' of 3.75 mg/kg, MA was administered intermittently or acutely. The intermittent dosing aimed to mimic casual use of the drug during pregnancy while the acute dose mimicked a once off dose that in the clinical scenario most often occurs at the later stages of pregnancy (Della Grotta *et al.*, 2010). This study revealed that a single MA insult in late pregnancy or several doses throughout the entire pregnancy are not as impairing to offspring as chronic daily doses. Overall the developmental profile for both groups was comparable to the controls. These neonatal results show that the amount of drug insults (frequencies) are another key factor in determining the neonatal outcome and the magnitude of retardations observed.

Route

The next aspect of our animal model was to simulate the route of administration of MA use in humans, namely oral administration. Therefore, oral gavage was employed in all studies and a comparison study using the sc route highlighted some interesting findings. Maternal behaviour was monitored in the postnatal period in chapters 4 and 5. MA exposure when given acutely, intermittently or chronically (via oral gavage) did not alter maternal behaviour but when given via sc injections maternal care for the pups was compromised. Altered maternal care and behaviour in turn can have a deleterious consequence on the offspring development. Although impaired maternal behaviour has been reported previously with higher doses of MA (5 mg/kg sc, (Slamberova *et al.*, 2005a, Slamberova *et al.*, 2005b)) the present findings are significant as it is clear that the route of administration is jointly responsible for these effects. Route of administration again played a major role in determining the neonatal outcome. Chapter 5 examined the difference in offspring outcome after *in utero* exposure to MA via oral gavage or sc injection. It was shown that the sc route produced an augmented effect on neurodevelopmental retardations. Delivering the drug via sc also resulted in unique neurodevelopmental retardations that were not observed with this dose when given via oral gavage. These findings draw into question previous studies and how translatable these results are. Previous studies using sc injections have shown that offspring exposed *in utero* to MA are susceptible in adulthood to increased anxiety-like behaviour, compulsivity, impulsivity, motivation for reward, amphetamine- and morphine-seeking behaviour and seizure occurrences (Slamberova, 2005, Slamberova *et al.*, 2015, Lloyd *et al.*, 2013, Slamberova *et al.*, 2012b). Yet, the long-term effects of MA exposure mentioned here are most likely correlated to the higher dose of MA employed

combined with the exaggerated outcome that accompanies the sc route and this may not transpire clinically.

Stages of life

An important feature of our model is the monitoring of development and behaviour throughout life including the neonatal, adolescent and adult stages of life. As mentioned above, many long-term effects of *in utero* MA have been reported but the translatability of these results can be questioned. Chapters 2 to 5 aimed to elucidate the neonatal outcomes and in order to determine if prenatal or postnatal MA exposure is in fact associated with long-term effects, this thesis also investigated behaviour in the adult offspring after the various MA exposure patterns employed in these chapters. An array of behavioural tests were used to examine changes in cognition, depressive-like behaviour, anxiety-like behaviour, locomotor activity and also responses to psychopharmacological challenges. Overall the study confirmed that perturbations in behaviour of these offspring are still present in adulthood with the most significant finding being increased depressive-like behaviour in the FST. This has not been previously reported as most studies have focused on cognitive performance in tests such as the MWM (Schutova *et al.*, 2008, Hruba *et al.*, 2010, Macuchova *et al.*, 2013, Schutova *et al.*, 2009a) but no significant effects were found for the MWM in the present work. Thus, it is likely that a higher dose is required to lead to enduring cognitive deficits in adulthood. The novel finding of depressive-like behaviour however, warrants further investigation. The chronic prenatal MA treatment with 3.75 mg/kg is the only exposure group to show this behaviour but these were noted in the male offspring of one experiment and the female offspring of another. The reason for this is most likely due to the fact that the first experiment

looked at behaviour in the re-exposure test (swim) while the second experiment looked at behaviour in the first exposure test (preswim test). This was due to the use of DMI dosing (sub-acute dosing) in the second experiment. Use of the preswim as an indication of depressive-like behaviour has been employed previously and is referred to as a modified FST (Cryan *et al*., 2005a, Cryan *et al*., 2005b, Lucki, 1997). However, in order to confirm these depressive-like findings future studies should consider the inclusion of other behavioural tests such as the saccharin/sucrose preference test.

Reproducibility/Variability

Predictive validity is essential when developing an animal model. In order to predict the outcome clinically, the preclinical model should be robust with findings that are consistent and reproducible. Overall, regarding the maternal outcome, each study is comparable and results are reproducible showing the robustness of this clinically relevant animal model. The neonatal findings, when compared across chapters, are consistent and reproducible with regard to all somatic developmental parameters. With regard to the behavioural neonatal findings more variation is notable when we consider the control groups across the various studies. Although variation can be expected between experiments, chapter 4 showed the most variation in baseline behaviour. For example in the negative geotaxis test on PND 9, 0% of control males performed this test. This is in contrast to the control males in other studies which on average have 50% of pups performing this test on this PND. This variation however is most likely due to the small sample size in chapter 4 and highlights the importance of having a higher number of litters to produce a robust result. The consistency and

robustness of our model is again seen in adulthood with similar results reproduced in each study.

Future Application

It is our belief that this preclinical animal model could be employed for other psychoactive drugs. Other psychoactive drugs such as the stimulants AMP, MDMA and mephedrone could also be applied to the model presented in this thesis and this would reveal important, translatable information related to these drugs and their effects when abused during pregnancy or lactation. The failure to mimic the clinical scenario is a common theme among the preclinical literature and so the aspects of our model could be applied to many psychoactive drugs in order to ensure construct, face and predictive validity. Although our extensive literature review and our research to date has been focused on MA, the concepts discussed in this thesis should highlight the importance of translatability from preclinical to clinical and reverse translatability from clinical to preclinical.

Causation

To the best of my knowledge, no studies have attempted to unearth the molecular mechanisms that are responsible for the developmental changes observed in both the neonatal and adult offspring. Enhanced oxidative stress has shown to be correlated with MA use preclinically in adult rats and clinically in adult abusers. In chapter 7 therefore, attempts were made to correlate the neonatal and adult neurodevelopmental deficits (found previously in our validated animal model) with alterations in oxidative stress. The present study failed to find any relationship between these parameters although it was shown that parturition and age amplified

oxidative stress. This study however is not without its limitations. Due to unforeseen circumstances not all oxidative stress assays (TBARS, GPx) were performed for non-pregnant and offspring samples. These additional assays may have revealed fluctuations in oxidative stress that were indiscernible in the assays performed. Although the offspring oxidative stress was assessed at various time points, oxidative stress in the dams was measured only at a single time point and after one hour of their last MA injection. Future work would need to assess a variety of time points after the last injection which was discussed further in chapter 1 II. A further limitation of the present study was the inability to investigate the neurotransmitter systems and this may have revealed changes in the DA-ergic system which are thought to trigger oxidative stress. Immunohistochemistry techniques to investigate DA neurons may have revealed some underlying trigger for these neurodevelopmental deficits but this was beyond the scope of the present thesis. Due to the lack of significant findings, oxidative stress was not examined for the other investigations (chapters 2, 4, 5).

Conclusion

The work presented in this thesis was carried out with the aim of determining the developmental, behavioural and neurochemical effects of early life exposure to MA using a clinically relevant animal model. This work has greatly contributed to our understanding of the area by providing an in-depth assessment of the different experimental parameters that affect offspring outcome. Table 8.1 highlights where we have come from, where we are and where we are going in terms of clinically relevant animal models with construct, face and predictive validity concepts.

	Past	Present	Future
Construct validity	-	·Route ·Causation	·Pharmacokinetic resemblance ·MA levels in blood, brain and breastmilk ·Causation
Face validity	· Adulthood behavioural assessments	·Neurodevelopmental parameters ·Dose ·Exposure timing ·Exposure frequency ·Stages of life	· Adulthood behavioural assessments ·Psychopharmacological drug challenges
Predictive validity	-	·Reproducibility ·Variability ·Robustness	·Screening for other psychoactive drugs

Table 8.2: Summary of construct, face and predictive validity concepts in past, present and future preclinical studies investigating the consequences of MA exposure during pregnancy or lactation in offspring.

The advancement from the previous literature to the achievements made in this thesis show the important contribution that this work has made. It can also guide us in future studies to see how we can build on this model and how we can employ this model further. A noteworthy advancement was the launching steps into the elucidation of putative molecular mechanisms underpinning the negative outcomes observed in these offspring. The findings in this thesis contribute to our understanding of the consequences of exposure to this drug of abuse in the neonatal and adult rat and may offer guidance clinically as to what might be expected when a mother abuses this drug. This in turn may also facilitate the development of new therapies targeted at both the mother and child.

8.2 Recommendations for future work

Methodological

- The levels of MA and its metabolites in the blood, brain and breastmilk of the mothers and offspring should be quantified in future studies. This would be most useful for the comparison study of gavage and sc routes of administration to assess if the disparity observed was in fact due to the higher amount of MA available.
- Pharmacokinetic resemblance to the clinical scenario has yet to be achieved preclinically for prenatal MA studies and future studies should aim to employ this if possible.

Behavioural

- Although the present thesis encompasses an array of behavioural parameters, future work should aim to incorporate more behavioural assessments in adulthood such as sucrose/saccharin preference and social interaction tests as well as evaluations of response to other psychopharmacological drug challenges such as cocaine and morphine. These paradigms may unmask further consequences of MA exposure that are not evident in the present thesis.

Neurochemical

- The precise mechanisms involved in these MA-induced deficits remain elusive. An unstudied area of research is still the neurochemical changes that occur as a result of MA exposure during pregnancy and lactation. In order to investigate oxidative stress further a broader spectrum of oxidative stress parameters and time points should be assessed which have been summarised in chapter 1.

- This ought to be accompanied by examination of the neurotransmitter systems (including DA, GABA and 5-HT) as these may draw attention to permanent changes in the brain that may prompt the behavioural alterations found.

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This bibliography relates to references from chapter 8.

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Appendix I:

Addendum

I.1 Addendum for Chapter 2

Abstract (Page 108)

In adulthood, no effects of prenatal MA exposure were found in the offspring in the open field, elevated plus maze or Morris water maze.

Materials and methods (Page 112)

After mating, all female rats were housed singly in plastic bottom cages with appropriate bedding material (Gold flake sawdust, Harlan, UK) and additional nesting materials (unbleached cotton and Nesteldown bedding, Petworld, Galway).

Materials and methods (Page 123)

Chi-square tests were run on both raw counts and percentages and as significance was the same, chi-square values for the percentage data are given since this is how the data is expressed.

I.2 Addendum for Chapter 3

Materials and methods (Page 157)

After mating, all female rats were housed singly in plastic bottom cages with appropriate bedding material (Gold flake sawdust, Harlan, UK) and additional nesting materials (unbleached cotton and Nesteldown bedding, Petworld, Galway).

Materials and methods (Page 163)

Chi-square tests were run on both raw counts and percentages and as significance was the same, chi-square values for the percentage data are given since this is how the data is expressed.

I.3 Addendum for Chapter 4

Materials and methods (Page 198)

Chi-square tests were run on both raw counts and percentages and as significance was not the same, chi-square values for the raw data are given.

I.4 Addendum for Chapter 5

Materials and methods (Page 221)

After mating, all female rats were housed singly in plastic bottom cages with appropriate bedding material (Pellets, 3Rs™, UK) and additional nesting materials (unbleached cotton and Nesteldown bedding, Petworld, Galway).

Materials and methods (Page 228)

Chi-square tests were run on both raw counts and percentages and as significance was the same, chi-square values for the percentage data are given since this is how the data is expressed.

Conclusion (Page 245)

At a lower dose of MA (2.5 mg/kg po) similar results were found with regards to ano-genital distance (McDonnell-Dowling *et al.*, 2014) however the reduction at this lower dose was not as great as the findings in the present study (11% reduction vs. 22% reduction).

Conclusion (Page 245)

In rats, to become a male *in utero* requires a chain of events initiated by activation of the Sry gene, which results in testis formation. ‘’Masculinization” is then driven by

testosterone produced by the fetal testis. Impaired fetal androgen action interferes with masculinization and can result in disorders of sexual differentiation (Welsh *et al.*, 2008). Therefore, in the present study it is thought that the testis are formed, but that the testosterone produced is hindered by MA exposure and results in delayed masculinization i.e. smaller ano-genital distance.

Appendix II:

Supplementary Results

II.1 Supplementary Results for Chapter 2

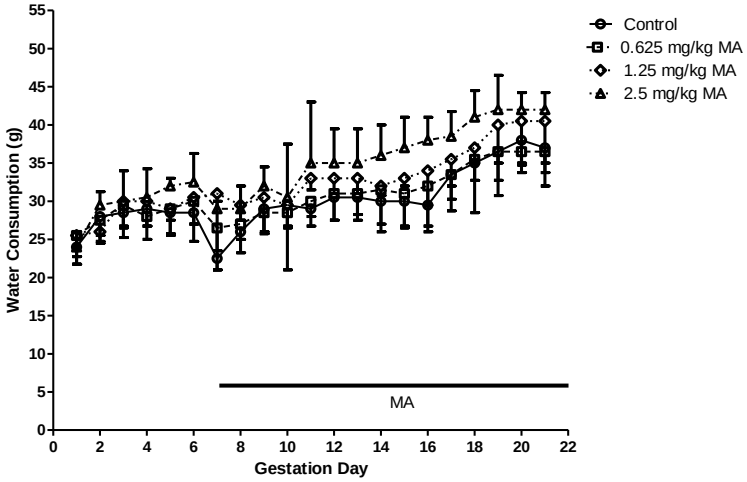

Figure A.1: Maternal water consumption during gestation. Water consumption for control, 0.625 mg/kg MA, 1.25 mg/kg MA, 2.5 mg/kg MA groups for each day of gestation (n=10/group). Line indicates dosing period. Data are expressed as Mean±SD; MA, methamphetamine.

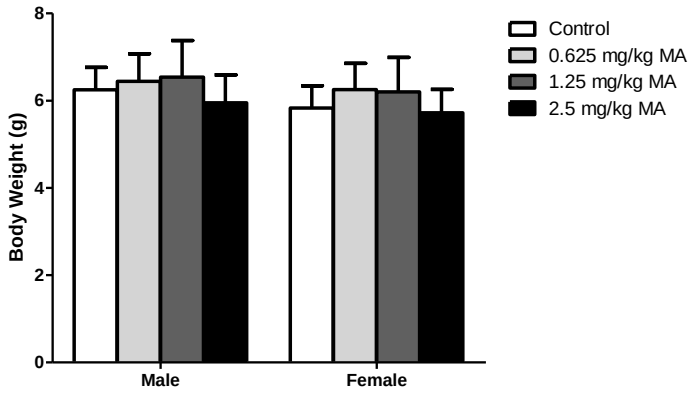

Figure A.2: Rat pup birth weights. Birth weights of male and female pups for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=32-40/group). Data are expressed as Mean+SD; MA, methamphetamine.

Group	No. of Pups/Litter	Pups Stillborn	Pups Found Dead	Pups Eaten	Total Deaths
Control	14 ± 3	0 ± 0	0 ± 1	0 ± 0	1 ± 1
0.625 mg/kg	14 ± 3	0 ± 0	0 ± 1	0 ± 0	0 ± 1
1.25 mg/kg	14 ± 3	0 ± 0	0 ± 1	0 ± 0	0 ± 1
2.5 mg/kg	14 ± 3	0 ± 0	0 ± 1	0 ± 0	1 ± 4

Table A.1: Litter observations. Litter observations at birth and in the postnatal period for control, 0.625 mg/kg MA, 1.25 mg/kg MA, 2.5 mg/kg MA groups (n=10/group). Data are expressed as Mean±SD; MA, methamphetamine.

Drug	PND 12 (%)	PND 17 (%)
Male		
Control	25	95
0.625 mg/kg	30	95
1.25 mg/kg	12	59
2.5 mg/kg	11	67
Female		
Control	20	80
0.625 mg/kg	30	85
1.25 mg/kg	24	71
2.5 mg/kg	11	72

Table A.2: Rat pup air righting. Air righting for male and female pups on PND 2, 3, 4 and 5 for control, 0.625 mg/kg MA, 1.25 mg/kg MA and 2.5 mg/kg MA (n=34-40/group). Data are expressed as percentage of pups that complete test; MA, methamphetamine.

II.2 Supplementary Results for Chapter 3

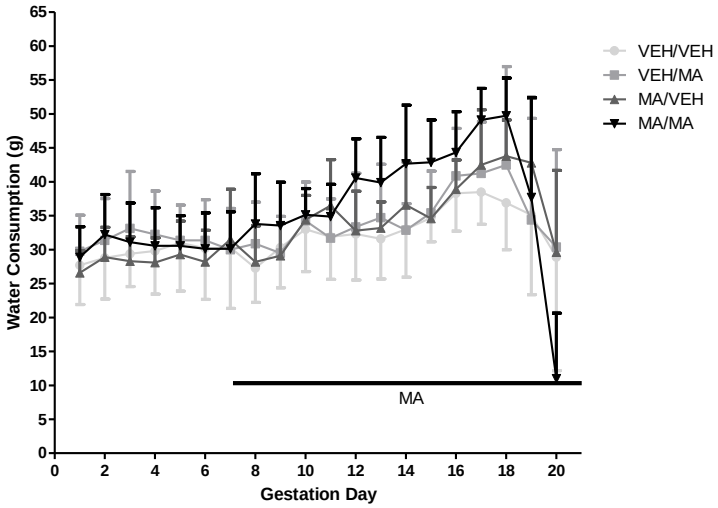

Figure A.3: Maternal water consumption during gestation. Water consumption for each day of gestation (n=8-10/group). Line indicates dosing period. Data are expressed as Mean±SD.

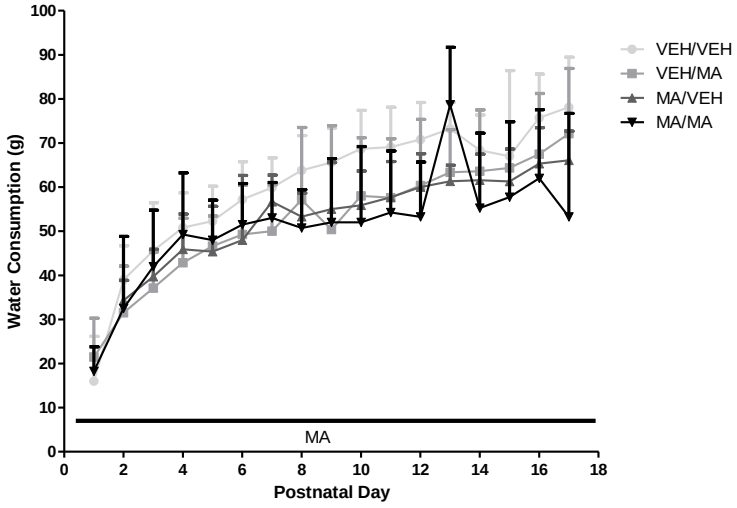

Figure A.4: Maternal water consumption during lactation. Food consumption for each day of lactation (n=4-10/group). Line indicates dosing period. Data are expressed as Mean±SD.

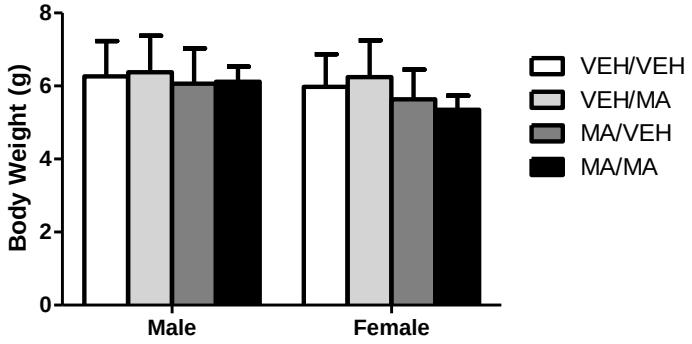

Figure A.5: Birth weights. Birth weights for male and female pups (n=4-10/group). Data are expressed as Mean+SD.

Drug	PND 3 (mm)
Male	
VEH/VEH	3.3 ± 0.4
VEH/MA	3.2 ±0.5
MA/VEH	3.4 ± 0.5
MA/MA	3.3 ±0.7
Female	
VEH/VEH	1.9 ± 0.2
VEH/MA	1.7 ± 0.2
MA/VEH	1.9 ± 0.4
MA/MA	1.6 ± 0.1

Table A.3: Ano-genital distance. Ano-genital distance for male and female pups on PND 3 (n=4-10/group). Data are expressed as Mean±SD.

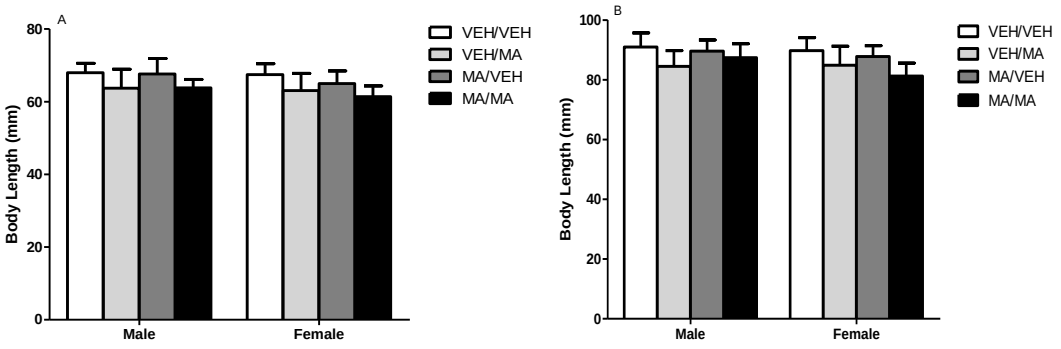

Figure A.6: Body length. Body length for male and female pups on (A) PND 7 and (B) PND 14 (n=4-10/group). Data are expressed as Mean+SD.

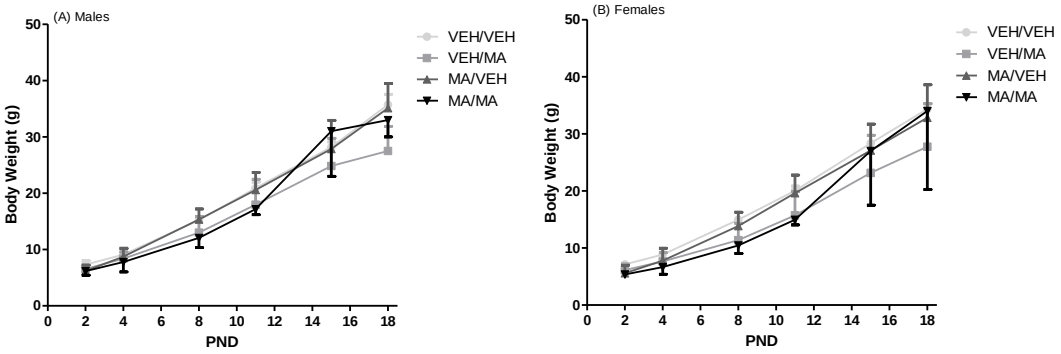

Figure A.7: Neonatal body weight. Body weight from PND 2-18 for (A) male and (B) female pups (n=4-10/group). Data are expressed as Mean±SD.

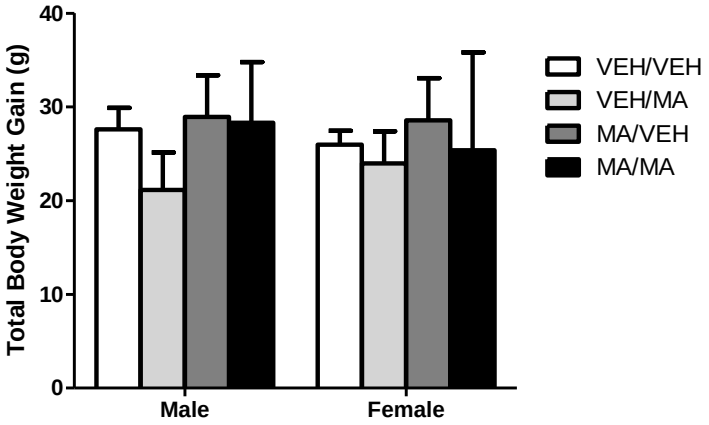

Figure A.8: Neonatal body weight gain. Body weight gain in the neonatal period (PND 1-18) for male and female pups (n=4-10/group). Data are expressed as Mean+SD.

II.3 Supplementary Results for Chapter 4

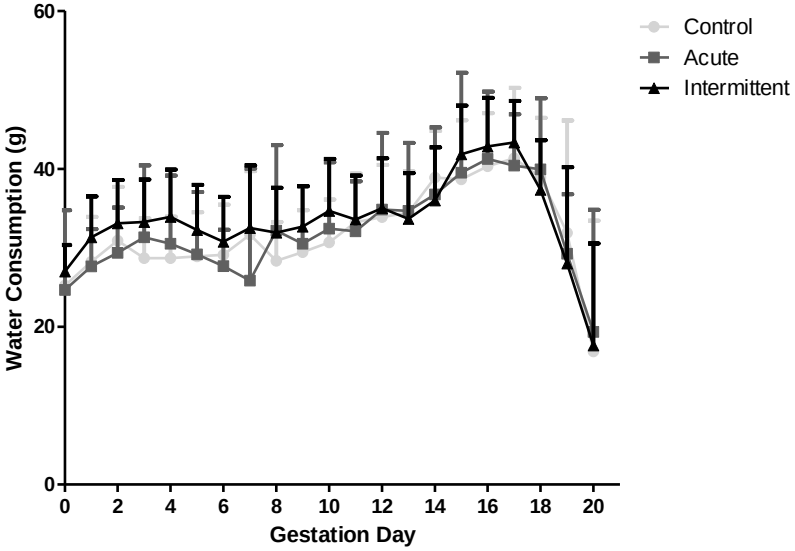

Figure A.9: Maternal water consumption during gestation. Water consumption for each day of gestation (n=9-12/group). Data are expressed as Mean±SD.

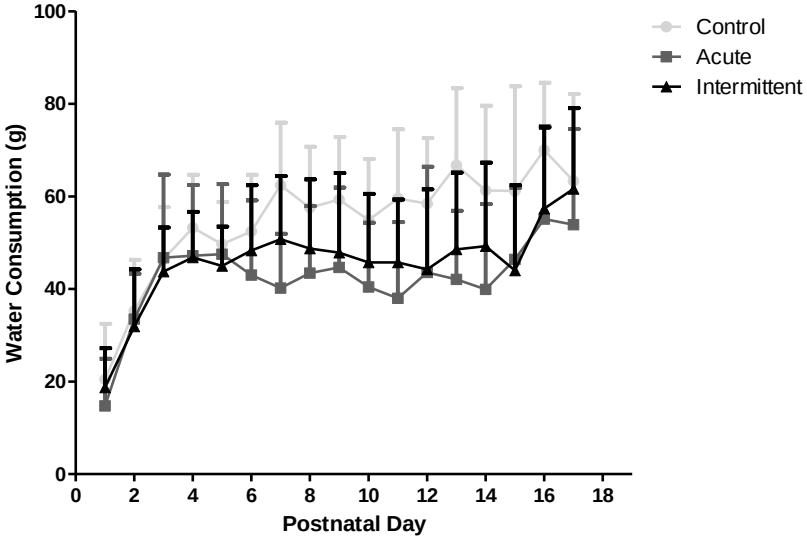

Figure A.10: Maternal water consumption during lactation. Water consumption for each day of lactation (n=5-6/group). Data are expressed as Mean±SD.

	Maternal activities				
Drug	**Active Nursing**	**Total Nursing**	**In nest**	**In contact with pups**	**Grooming pups**
Control	5 ± 1	6 ± 1	7 ± 2	7 ± 2	3 ± 1
Acute	5 ± 1	6 ± 1	7 ± 2	7 ± 2	3 ± 1
Intermittent	5 ± 1	6 ± 1	7 ± 2	7 ± 2	3 ± 1

Table A.4: Maternal behaviour in the observational test. Maternal observations in the postnatal period (n=5-6 dams/group). Data are expressed as Mean±SD.

	Maternal activities		
Drug	**Carry first pup**	**First pup in nest**	**All pups in nest**
Control	7 (2 - 90)	27 (9 – 91)	104 (50 – 315)
Acute	6 (2 – 43)	7 (4 – 40)	39 (28 – 84)
Intermittent	4 (3 – 9)	5 (4 – 10)*	32 (22 – 56)

Table A.5: Maternal behaviour in the retrieval test. Maternal observations in the postnatal period (n=5-6 dams/group). Data are expressed as Median and Interquartile range in seconds, *p<0.05 vs. control.

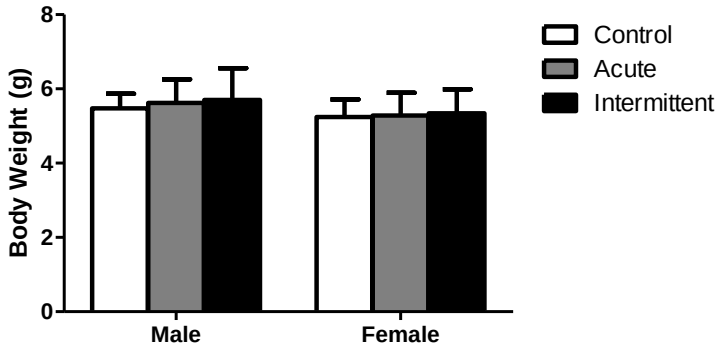

Figure A.11: Birth weights. Birth weights for male and female pups (n=10/group). Data are expressed as Mean+SD.

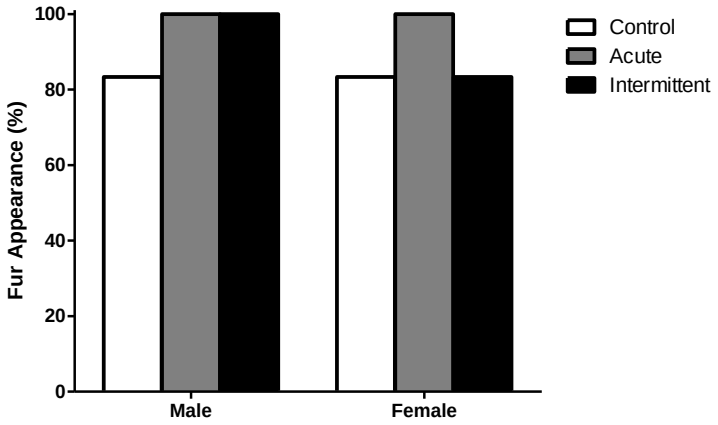

Figure A.12: Fur appearance. Percentage of pups with fur for male and female pups on PND 3 (n=5-6/group). Data are expressed as percentage of pups with fur.

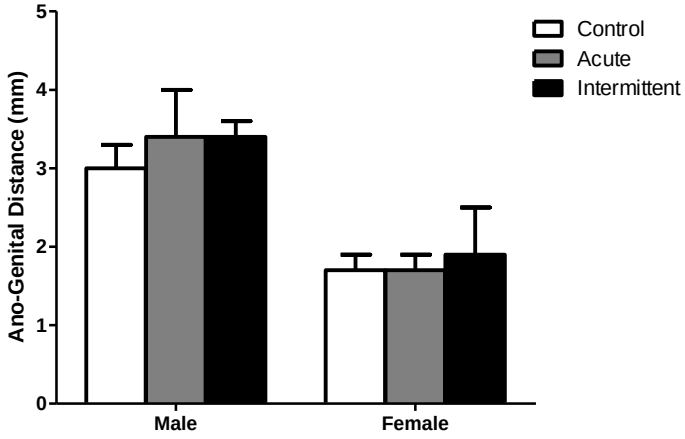

Figure A.13: Ano-genital distance. Ano-genital distance for male and female pups on PND 3 (n=5-6/group). Data are expressed as Mean+SD.

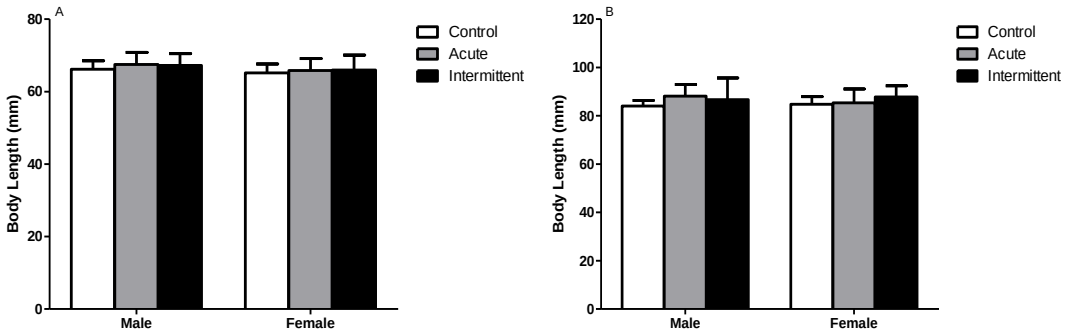

Figure A.14: Body length. Body length for male and female pups on (A) PND 7 and (B) PND 14 (n=5-6/group). Data are expressed as Mean+SD.

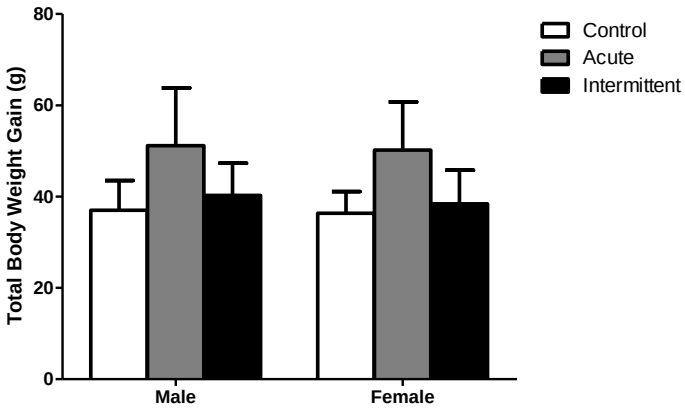

Figure A.15: Neonatal body weight gain. Neonatal body weight gain for male and female pups for PND 1-21 (n=5-6/group). Data are expressed as Mean+SD.

II.4 Supplementary Results for Chapter 5

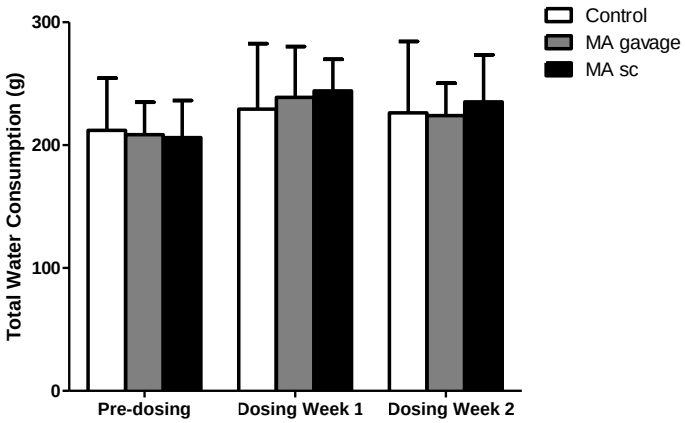

Figure A.16: Maternal water consumption during gestation. Total water consumption from the pre-dosing and dosing periods (n=10/group). Data are expressed as Mean+SD.

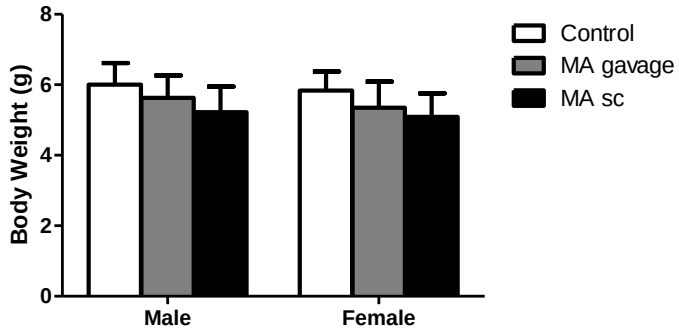

Figure A.17: Birth weights. Birth weights for male and female pups (n=10/group). Data are expressed as Mean+SD.

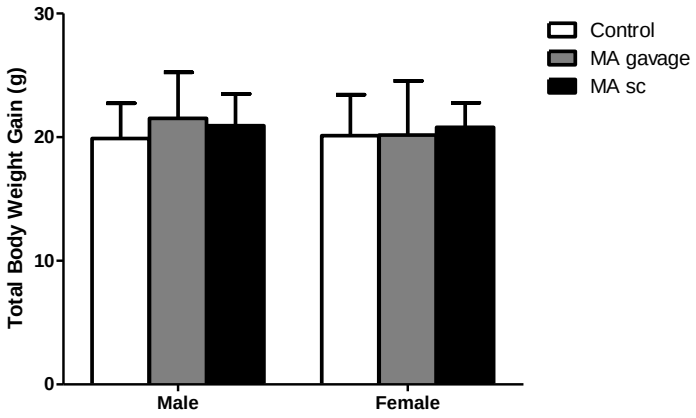

Figure A.18: Neonatal body weight gain. Body weight gain for male and female pups (n=10/group). Data are expressed as Mean+SD.

II.5 Supplementary Results for Chapter 6

Experiment 1: Dose Response

Drug	Distance moved (cm)	Velocity (cm/s)	Open Arm Time (%)	Open Arm Frequency (%)
Male				
Control	1502 ± 177	5 ± 1	27 ± 14	41 ± 11
0.625mg	1469 ± 233	5 ± 1	29 ± 14	47 ± 15
1.25mg	1439 ± 352	5 ± 1	27 ± 14	42 ± 11
2.5mg	1351 ± 393	5 ± 1	26 ± 15	43 ± 9
Female				
Control	1714 ± 310	6 ± 1	35 ± 27	42 ± 13
0.625mg	1548 ± 189	5 ± 1	38 ± 11	41 ± 7
1.25mg	1661 ± 112	6 ± 0	37 ± 10	49 ± 6
2.5mg	1677 ± 324	6 ± 1	36 ± 17	47 ± 20

Table A.6: Elevated plus maze. Parameters of the EPM for males and females on PND 56 (n=16-19/group). Data are expressed as Mean±SD.

Drug	Distance moved (cm)	Velocity (cm/s)
Male		
Control	2591 ± 404	9 ± 1
0.625mg	2275 ± 585	8 ± 2
1.25mg	2589 ± 473	9 ± 2
2.5mg	2218 ± 697	7 ± 2
Female		
Control	2560 ± 420	9 ± 1
0.625mg	2349 ± 287	8 ± 1
1.25mg	3158 ± 533	11 ± 2
2.5mg	2590 ± 439	9 ± 2

Table A.7: Open field. Parameters of the OF for males and females on PND 56 (n=16-19/group). Data are expressed as Mean±SD.

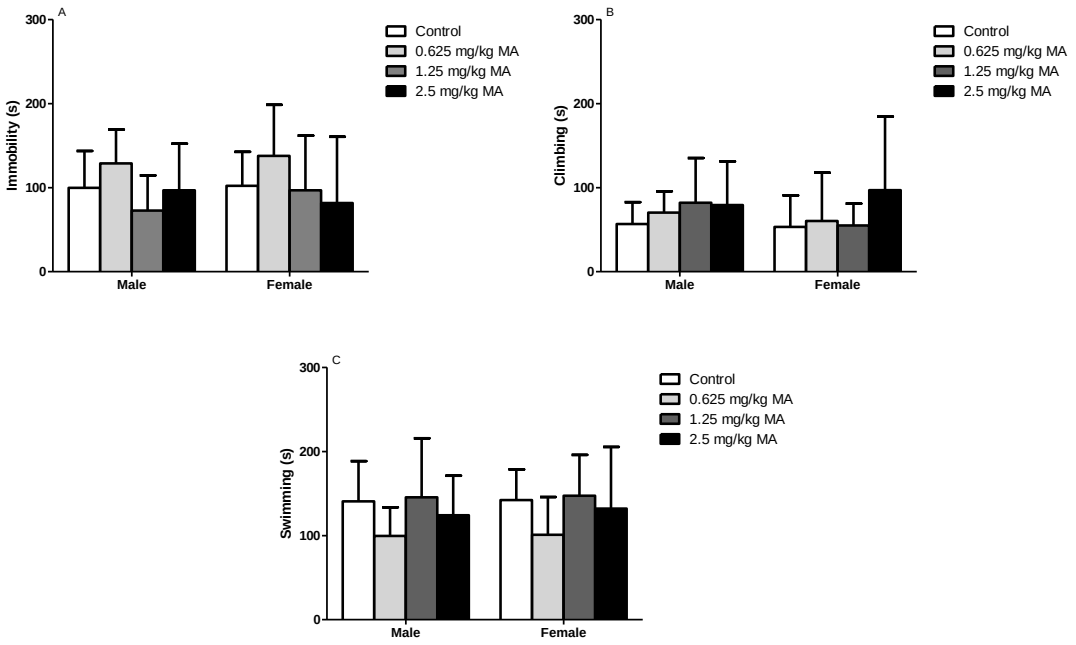

Figure A.19: Forced swim test. Time spent (A) immobile, (B) climbing and (C) swimming in the FST for male and females on PND 56 (n=16-19/group). Data are expressed as Mean+SD.

Drug	Distance moved (cm)	Velocity (cm/s)
Male		
Control	2019 ± 385	7 ± 1
0.625mg	2129 ± 381	7 ± 1
1.25mg	2133 ± 382	7 ± 1
2.5mg	2347 ± 594	8 ± 2
Female		
Control	2389 ± 471	8 ± 2
0.625mg	1902 ± 419	6 ± 1
1.25mg	2371 ± 404	8 ± 1
2.5mg	2308 ± 658	8 ± 2

Table A.8: Open field. Parameters of the OF for males and females on PND 85 (n=16-20/group). Data are expressed as Mean±SD.

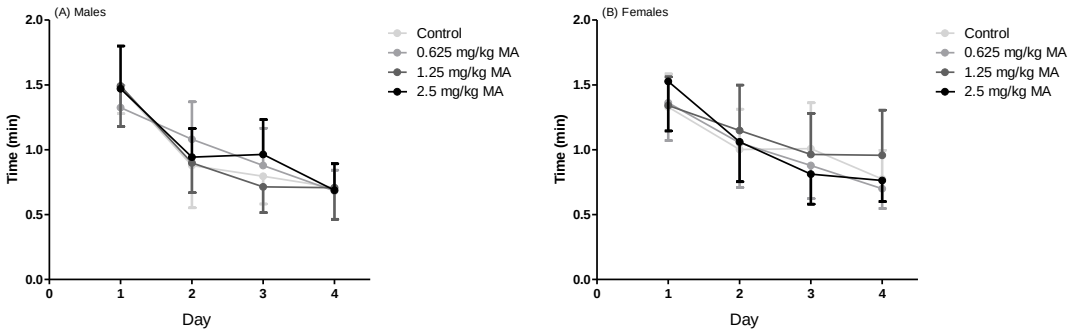

Figure A.20: Morris water maze. Time to find platform in the MWM for (A) males and (B) females on PND 85 (n=16-18/group). Data are expressed as Mean±SD.

Drug	Distance moved (cm)	Velocity (cm/s)	Open Arm Time (%)	Open Arm Frequency (%)
Male				
Control	1176 (1058-1477)	4 (4-5)	2 (0-7)	13 (0-18)
0.625mg	1367 (1178-1619)	5 (4-5)	4 (1-13)	16 (7-22)
1.25mg	1169 (1029-1322)	4 (3-4)	2 (0-23)	9 (0-25)
2.5mg	1248 (1148-1338)	4 (4-5)	2 (1-20)	20 (8-30)
Female				
Control	1528 (1201-1719)	5 (4-6)	15 (0-27)	24 (3-35)
0.625mg	1511 (1312-1575)	5 (4-5)	12 (6-16)	28 (17-37)
1.25mg	1559 (876-1741)	5 (3-6)	7 (4-31)	23 (20-38)
2.5mg	1570 (1316-1739)	5 (4-6)	18 (7-28)	35 (20-48)

Table A.9: Elevated plus maze. Parameters of the EPM for males and females on PND 113 (n=15-27/group). Data are expressed as Median and Interquartile range.

Drug	Distance moved (cm)	Velocity (cm/s)
Male		
Control	2193 ± 1167	9 ± 4
0.625mg	2064 ± 509	8 ± 3
1.25mg	1451 ± 1061	6 ± 5
2.5mg	1868 ± 476	8 ± 1
Female		
Control	1669 ± 914	7 ± 3
0.625mg	2207 ± 473	8 ± 2
1.25mg	1578 ± 1008	7 ± 5
2.5mg	1316 ± 750	6 ± 23

Table A.10: Open field. Parameters of the OF for males and females on PND 113 (n=14-21/group). Data are expressed as Mean±SD

Experiment 2: Prenatal and Postnatal

Drug	Distance moved (cm)	Velocity (cm/s)	Open Arm Time (%)	Open Arm Frequency (%)
Male				
VEH/VEH	1660 ± 246	6 ± 1	31 ± 16	30 ± 11
VEH/MA	1781 ± 183	8 ± 1	27 ± 17	29 ± 14
MA/VEH	1874 ± 334	10 ± 1	33 ± 13	37 ± 9
Female				
VEH/VEH	1764 ± 243	7 ± 1	34 ± 11	28 ± 8
VEH/MA	1816 ± 299	9 ± 1	33 ± 16	35 ± 16
MA/VEH	1831 ± 251	11 ± 1	31 ± 19	33 ± 12

Table A.11: Elevated plus maze. Parameters of the EPM for males and females on PND 57 (n=7-10/group). Data are expressed as Mean±SD.

Drug	Distance moved (cm)	Velocity (cm/s)	Inner Zone Duration (s)	Inner Zone Entries
Male				
VEH/VEH	2441 ± 516	8 ± 2	12 ± 5	16 ± 6
VEH/MA	2409 ± 479	8 ± 2	11 ± 6	16 ± 7
MA/VEH	2443 ± 530	8 ± 2	14 ± 8	21 ± 9
Female				
VEH/VEH	2360 ± 367	8 ± 1	10 ± 5	15 ± 6
VEH/MA	2698 ± 518	9 ± 2	13 ± 5	23 ± 14
MA/VEH	2351 ± 396	8 ± 1	8 ± 8	16 ± 2

Table A.12: Open field. Parameters of the OF for males and females on PND 57 (n=7-10/group). Data are expressed as Mean±SD.

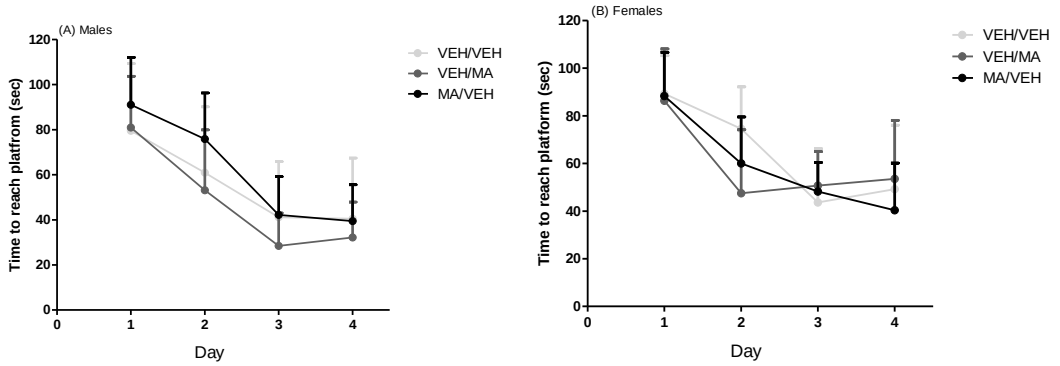

Figure A.21: Morris water maze. Time to find platform in the MWM on PND 57 for (A) males and (B) females (n=7-10/group). Data are expressed as Mean+SD.

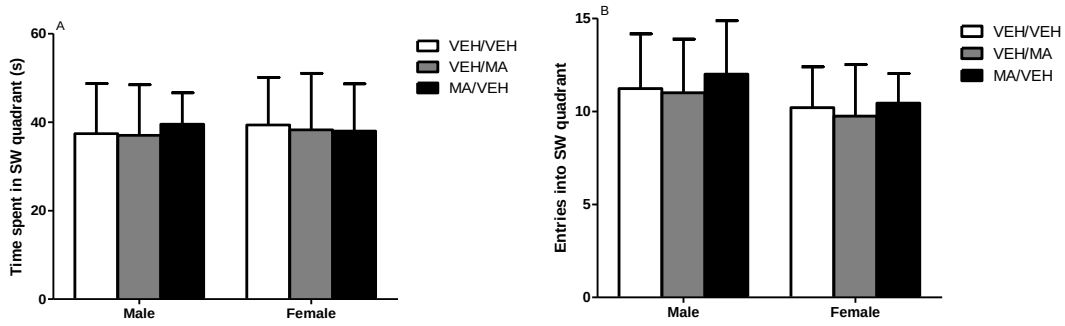

Figure A.22: Morris water maze. (A) Time spent in SW quadrant and (B) entries into SW quadrant in the MWM on PND 57 for males and females (n=7-10/group). Data are expressed as Mean+SD.

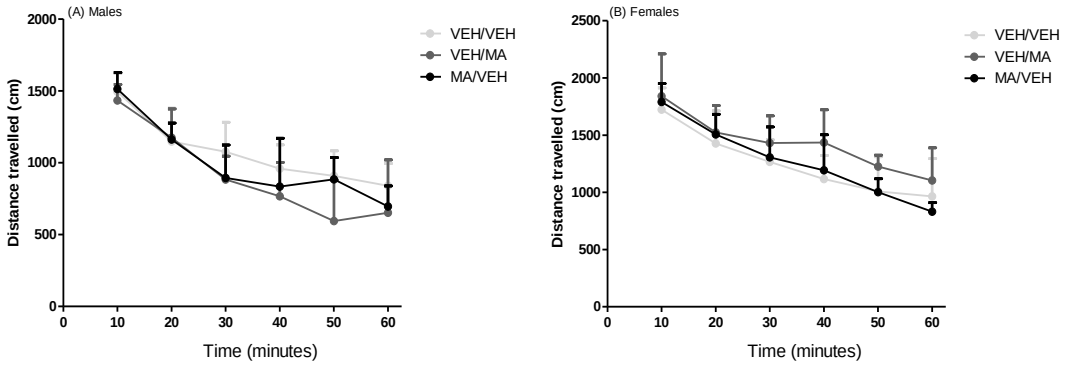

Figure A.23: Home cage activity. (A) Distance moved for males and (B) females in the HCA on PND 57 (n=6-10/group). Data are expressed as Mean+SD.

Drug	Distance moved (cm)	Velocity (cm/s)	Open Arm Time (%)	Open Arm Frequency (%)
Male				
VEH/VEH	1446 ± 314	5 ± 1	32 ± 14	28 ± 10
VEH/MA	1617 ± 201	5 ± 1	19 ± 15	21 ± 11
MA/VEH	1508 ± 246	5 ± 1	30 ± 20	34 ± 9
Female				
VEH/VEH	1682 ± 280	6 ± 1	24 ± 15	32 ± 12
VEH/MA	1985 ± 256	7 ± 1	44 ± 14	41 ± 15
MA/VEH	1843 ± 221	6 ± 1	46 ± 8	46 ± 6

Table A.13: Elevated plus maze. Parameters of the EPM for males and females on PND 85 (n=7-10/group). Data are expressed as Mean±SD.

Drug	Distance moved (cm)	Velocity (cm/s)	Inner Zone Duration (s)	Inner Zone Entries
Male				
VEH/VEH	2339 ± 296	8 ± 1	13 ± 13	16 ± 11
VEH/MA	2713 ± 513	9 ± 2	17 ± 17	18 ± 14
MA/VEH	2163 ± 568	7 ± 2	8 ± 6	12 ± 10
Female				
VEH/VEH	2783 ± 624	9 ± 2	13 ± 9	17 ± 7
VEH/MA	2814 ± 558	9 ± 2	14 ± 5	21 ± 11
MA/VEH	2744 ± 268	9 ± 1	13 ± 7	19 ± 7

Table A.14: Open field. Parameters of the OF for males and females on PND 85 (n=7-9/group). Data are expressed as Mean±SD.

Drug	Distance moved (cm)	Velocity (cm/s)	Open Arm Time (%)	Open Arm Frequency (%)
Male				
VEH/VEH	1411 ± 132	5 ± 0	26 ± 17	34 ± 16
VEH/MA	1671 ± 68	6 ± 0	29 ± 13	30 ± 6
MA/VEH	1560 ± 158	5 ± 1	21 ± 16	30 ± 19
Female				
VEH/VEH	1655 ± 186	6 ± 1	24 ± 14	30 ± 15
VEH/MA	1840 ± 294	6 ± 1	25 ± 16	35 ± 11
MA/VEH	1652 ± 370	6 ± 1	25 ± 13	26 ± 11

Table A.15: Elevated plus maze. Parameters of the EPM for males and females on PND 113 (n=5-10/group). Data are expressed as Mean±SD.

Drug	Distance moved (cm)	Velocity (cm/s)	Inner Zone Duration (s)	Inner Zone Entries
Male				
VEH/VEH	2396 ± 426	8 ± 1	9 ± 6	12 ± 8
VEH/MA	2526 ± 394	8 ± 1	16 ± 8	18 ± 8
MA/VEH	2389 ± 394	8 ± 1	7 ± 2	11 ± 7
Female				
VEH/VEH	2752 ± 426	9 ± 1	12 ± 7	18 ± 7
VEH/MA	2912 ± 659	10 ± 2	17 ± 10	21 ± 10
MA/VEH	2390 ± 213	8 ± 1	16 ± 8	21 ± 7

Table A.16: Open field. Parameters of the OF for males and females on PND 113 (n=6-9/group). Data are expressed as Mean±SD.

Experiment 3: Intermittent Exposure

Drug	Distance moved (cm)	Velocity (cm/s)	Open Arm Time (%)	Open Arm Frequency (%)
Male				
Control	1494 (1237-1773)	5 (4-6)	30 (14-45)	33 (14-42)
Acute	1401 (1208-1567)	5 (4-5)	24 (11-27)	25 (12-37)
Intermittent	1705 (1404-1762)	6 (5-6)	28 (16-41)	29 (26-40)
Female				
Control	1891 (1740-1970)	6 (6-7)	37 (33-42)	35 (32-42)
Acute	1619 (1247-1854)	5 (4-6)	37 (32-49)	38 (27-41)
Intermittent	1797 (1541-2322)	6 (5-8)	45 (34-51)	38 (32-40)

Table A.17: Elevated plus maze. Parameters of the EPM for males and females on PND 56 (n=4-7/group). Data are expressed as Median and Interquartile Range.

Drug	Distance moved (cm)	Velocity (cm/s)	Inner Zone Duration (%)	Inner Zone Entries (%)
Male				
Control	2450 (2292-2901)	8 (8-10)	9 (5-14)	50 (49-50)
Acute	2415 (1509-2810)	8 (5-9)	9 (7-22)	50 (49-50)
Intermittent	2450 (2182-2589)	8 (7-9)	13 (6-13)	50 (49-50)
Female				
Control	2849 (2600-3319)	10 (9-11)	7 (3-15)	50 (46-51)
Acute	2844 (2247-3454)	10 (8-12)	18 (6-29)	50 (50-51)
Intermittent	2525 (1862-3377)	8 (6-11)	7 (3-18)	50 (47-50)

Table A.18: Open field. Parameters of the OF for males and females on PND 56 (n=4-7/group). Data are expressed as Median and Interquartile Range.

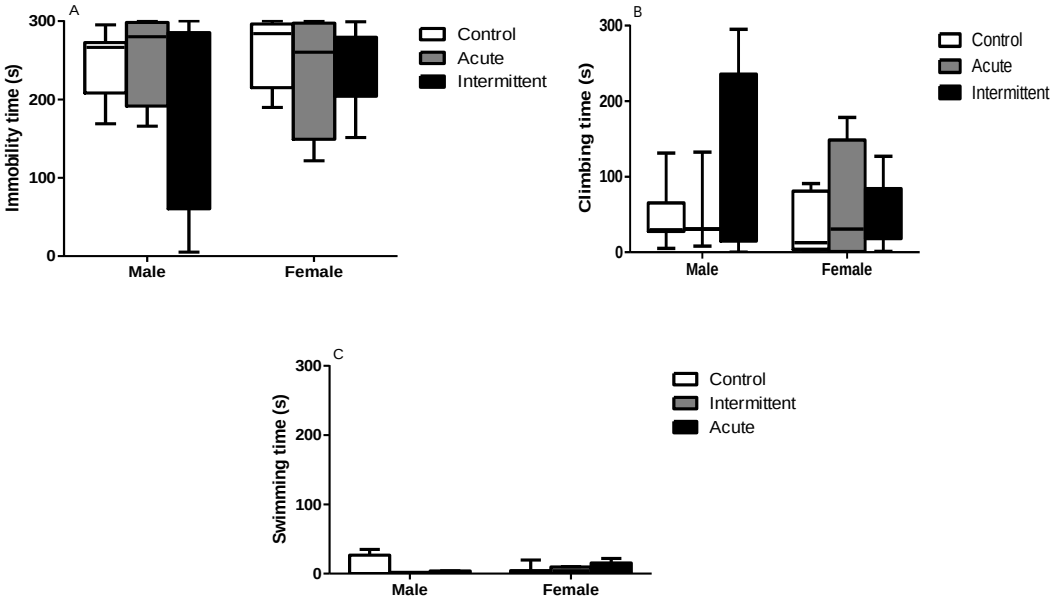

Figure A.24: Forced swim test. Parameters of the FST including time spent (A) immobile, (B) climbing and (C) swimming for males and females on PND 56 (n=4-7/group). Data are expressed as Median, Interquartile range, min and max.

Experiment 4: Route of Administration

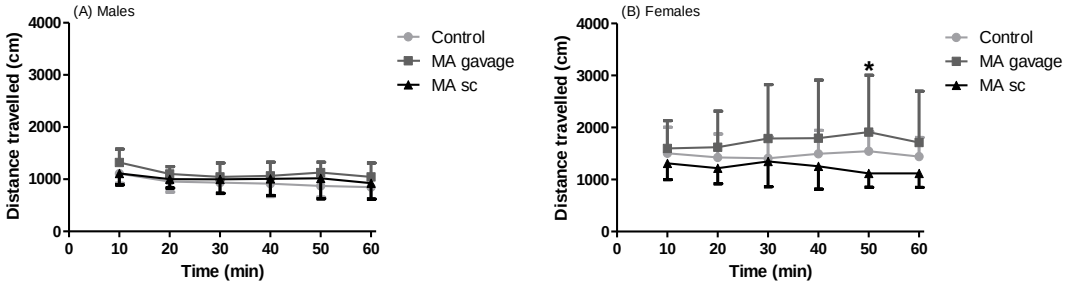

Figure A.25: Home cage activity. Distance travelled in 10 min time bins in the HCA for (A) males and (B) females on PND 56 (n=6-7/group). Data are expressed as Means±SD; *p<0.05 vs. MA sc.

II.6 Supplementary Results for Chapter 7

Experiment 1: Maternal Oxidative Stress

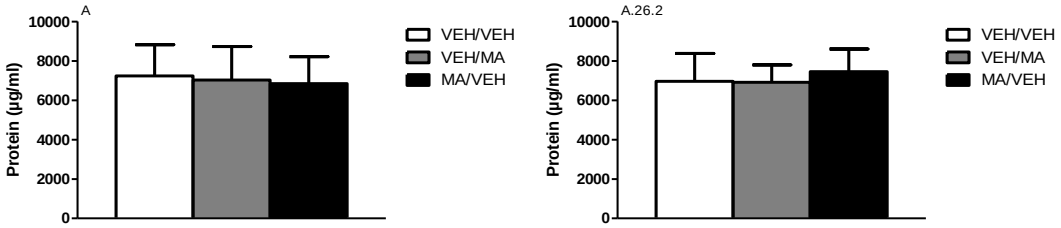

Figure A.26: Maternal protein concentration. Protein concentrations in the (A) striatum and (B) frontal cortex (n=11/group). Data are expressed as Mean+SD.

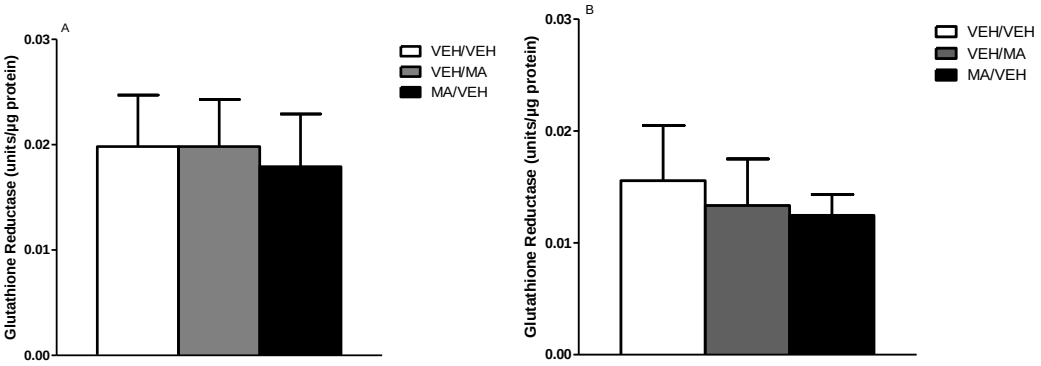

Figure A.27: Maternal glutathione reductase concentration. Glutathione Reductase concentrations in the (A) striatum and (B) frontal cortex (n=9-11/group). Data are expressed as Mean+SD.

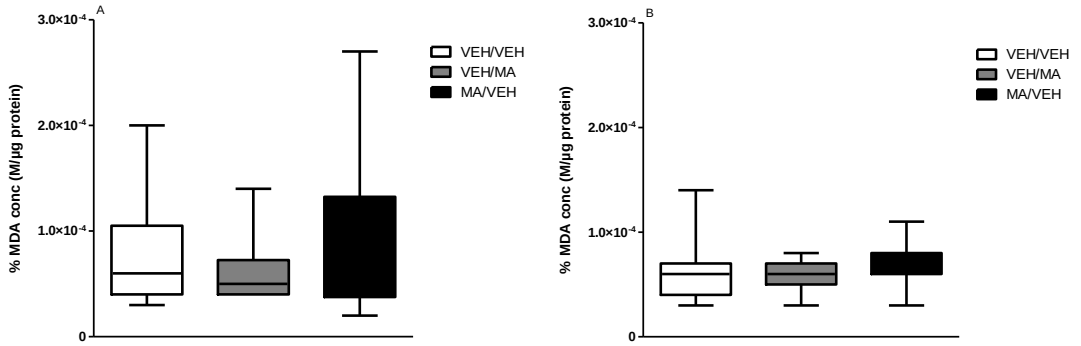

Figure A.28: Maternal malondialdehyde concentration. MDA concentrations in the (A) striatum and (B) frontal cortex (n=9-11/group). Data are expressed as Median, Interquartile range, min and max.

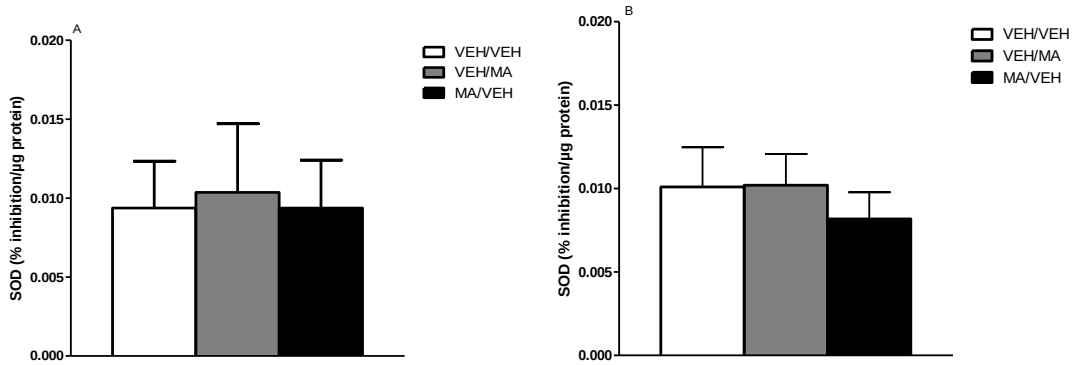

Figure A.29: Maternal SOD activity. SOD activity in the (A) striatum and (B) frontal cortex (n=10-11/group). Data are expressed as Mean+SD.

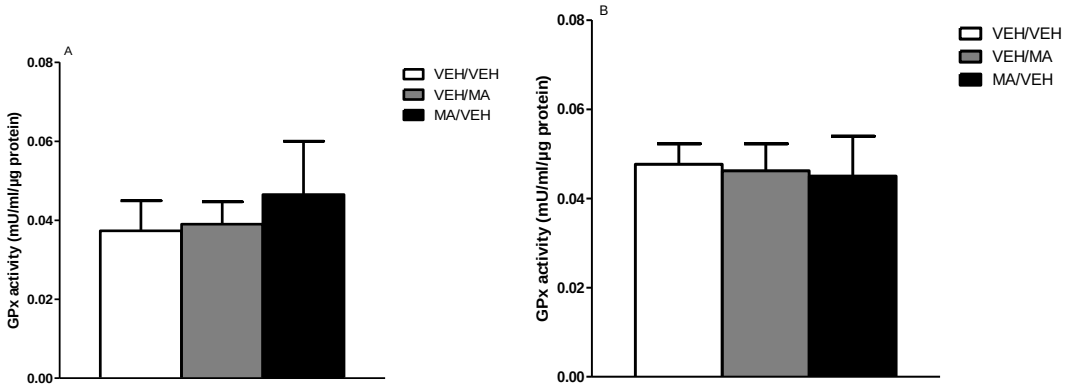

Figure A.30: Maternal glutathione peroxidase activity. Glutathione Peroxidase activity in the (A) striatum and (B) frontal cortex (n=5-6/group). Data are expressed as Mean+SD.

Experiment 3: Non-Pregnant Oxidative Stress

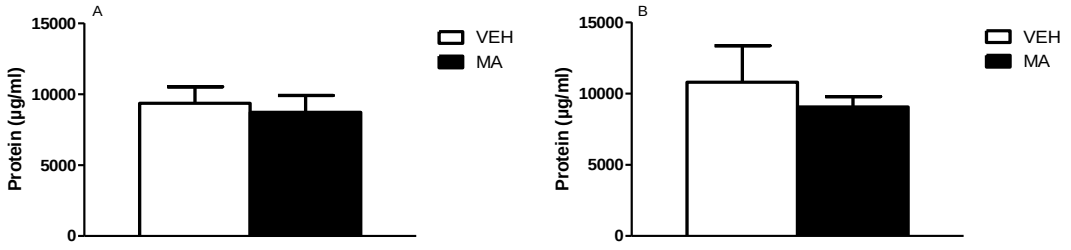

Figure A.31: Non-Pregnant protein concentration. Protein concentrations in the (A) striatum and (B) frontal cortex for VEH (n=3) and MA (n=6). Data are expressed as Mean+SD.

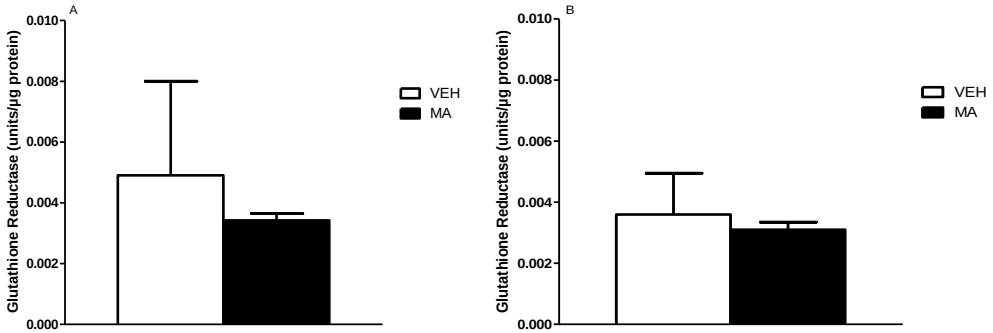

Figure A.32: Non-Pregnant glutathione reductase concentration. Glutathione Reductase concentrations in the (A) striatum and (B) frontal cortex for VEH (n=3) and MA (n=6). Data are expressed as Mean+SD.

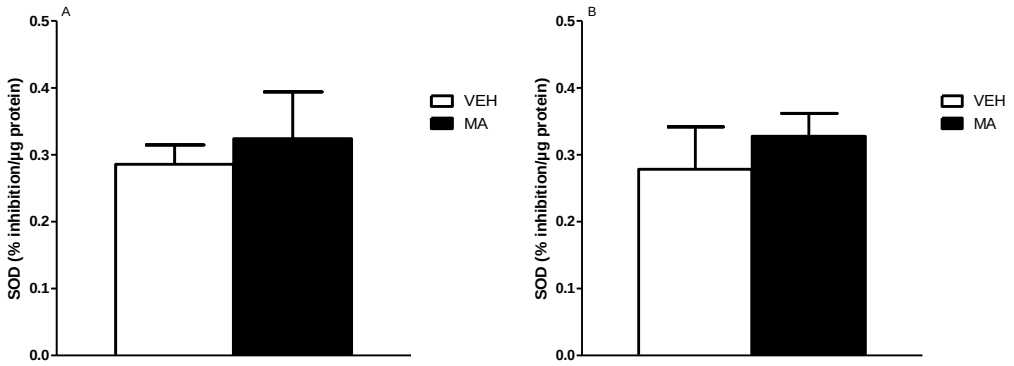

Figure A.33: Non-Pregnant SOD activity. SOD activity in the (A) striatum and (B) frontal cortex for VEH (n=3) and MA (n=6). Data are expressed as Mean+SD.

II.7 Supplementary Results

Parameter	Chapter 2	Chapter 3	Chapter 4	Chapter 5
Maternal body weight	No	Yes	Yes	Yes
Maternal food consumption	No	Yes	No	Yes
Maternal water consumption	No	No	No	No
Maternal behaviour	-	-	No	Yes
Maternal gestation length	No	No	No	No
Litter characteristics	No	No	Yes	Yes
Neonatal death	No	Yes	Yes	No
Birth weights	No	No	No	No
Ano-genital distance	Yes	No	No	Yes
Body length	Yes	No	No	Yes
Pinna unfolding	No	Yes	No	Yes
Fur appearance	Yes	Yes	No	Yes
Eye opening	Yes	Yes	No	Yes
Surface righting	Yes	Yes	Yes	Yes
Air righting	No	-	-	-
Negative geotaxis/ Inclined planet	Yes	Yes	No	Yes
Forelimb grip	Yes	Yes	No	Yes
Offspring body weight	No	No	No	No

Table A.19: Summary table of significant results found in results chapters 2, 3, 4 and 5. A yes answer means that significant effects of MA were found. An empty space (-) means that this was not performed for this chapter.

Parameter	Chapter 2	Chapter 3	Chapter 4	Chapter 5
Birth weights	Yes	No	No	No
Ano-genital distance	Yes	Yes	Yes	Yes
Body length	No	No	No	No
Pinna unfolding	No	Yes	No	Yes
Fur appearance	No	No	No	No
Eye opening	Yes	Yes	No	Yes
Surface righting	Yes	Yes	Yes	Yes
Air righting	No	-	-	-
Negative geotaxis/ Inclined plane	No	No	No	No
Forelimb grip	Yes	Yes	No	Yes
Offspring body weight	No	No	No	No

Table A.20: Summary table of significant sex effects found in results chapters 2, 3, 4 and 5. A yes answer means that significant effects of sex were found. An empty space (-) means that this was not performed for this chapter.

Appendix III:

Study Materials

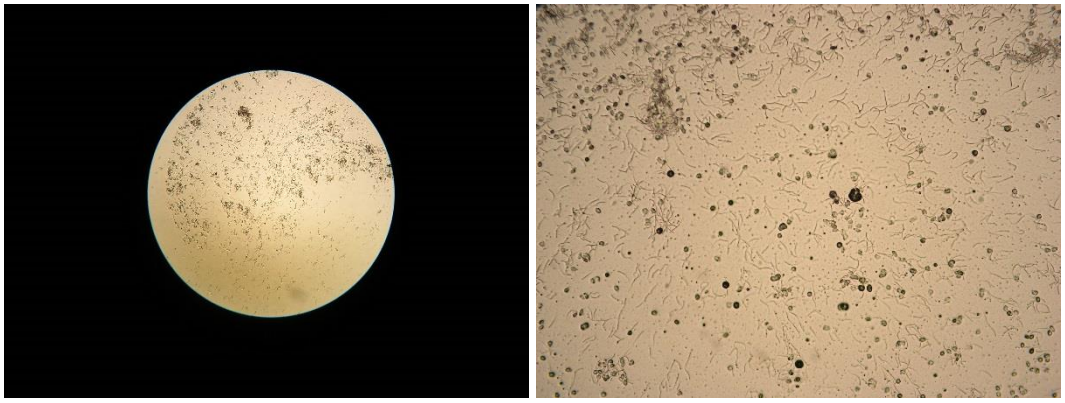

Figure B.1: Rat sperm in a vaginal smear seen under a microscope at x10 and x100.

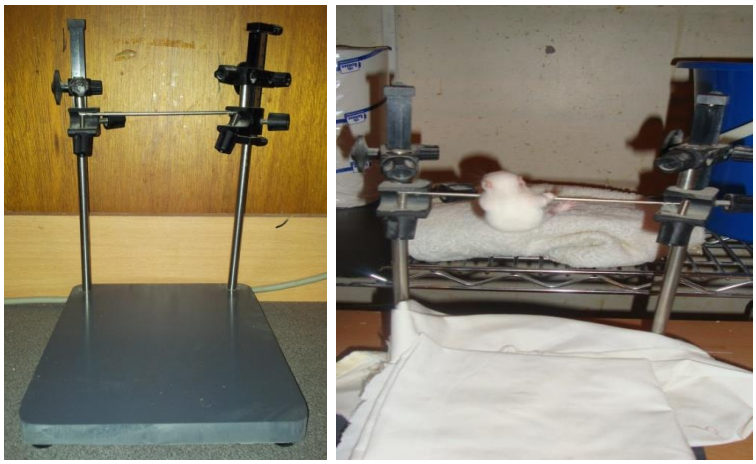

Figure B.2: Forelimb grip apparatus.

Figure B.3: Negative geotaxis/inclined plane apparatus.

Figure B.4: Maternal behaviour in the observation test in the home cage.

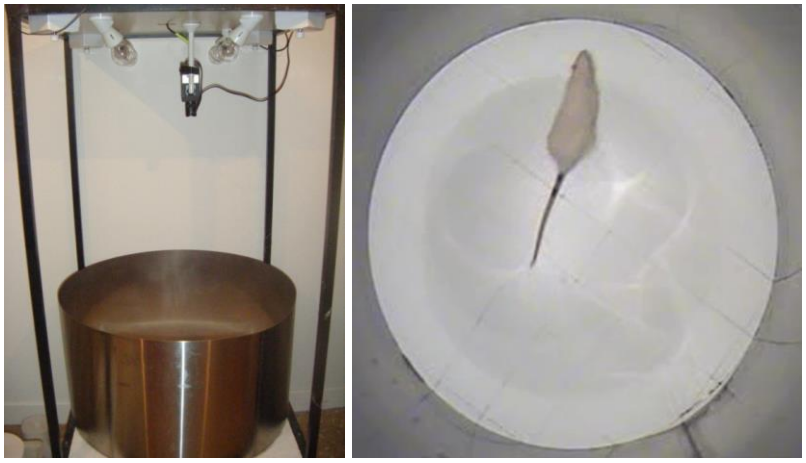

Figure B.5: Open field apparatus.

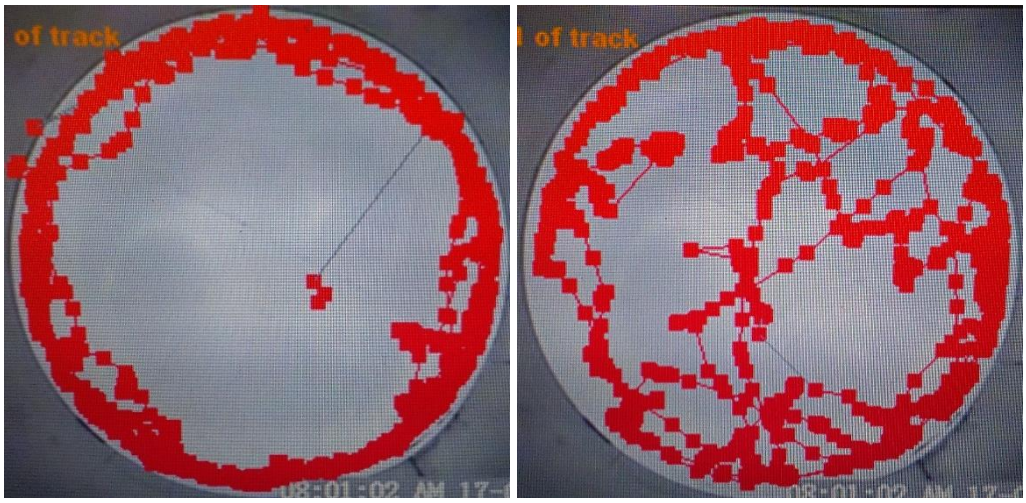

Figure B.6: Tracked behaviour of the rat in the open field test using Ethovision software.

Figure B.7: Elevated plus maze apparatus.

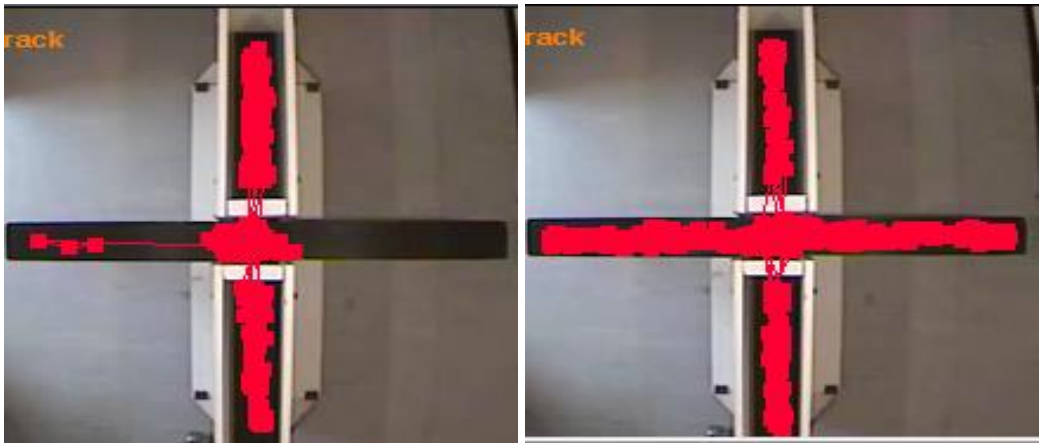

Figure B.8: Tracked behaviour of the rat in the elevated plus maze using Ethovision software.

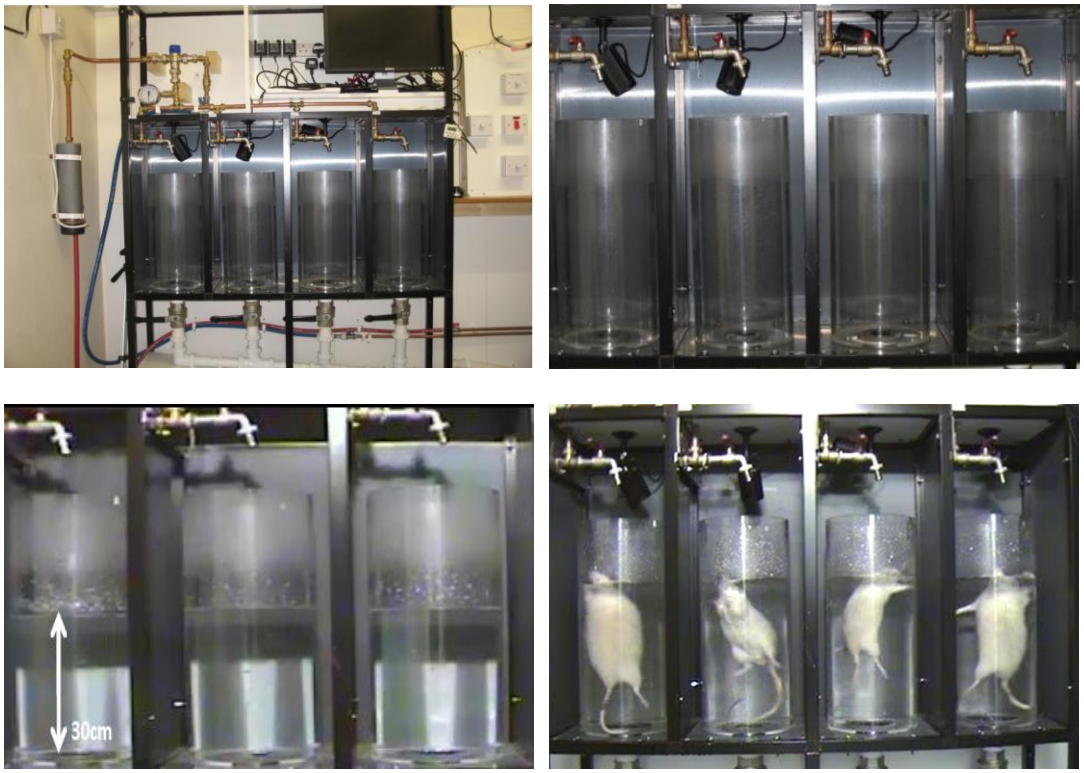

Figure B.9: Forced swim test apparatus.

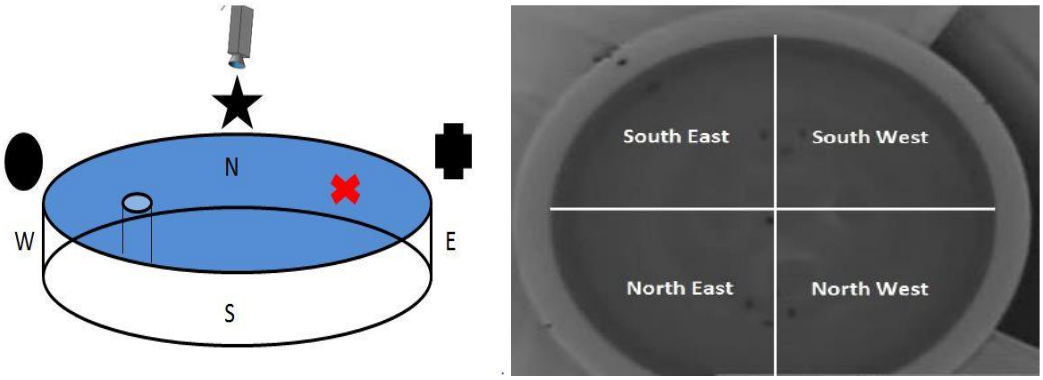

Figure B.10: Morris water maze apparatus.

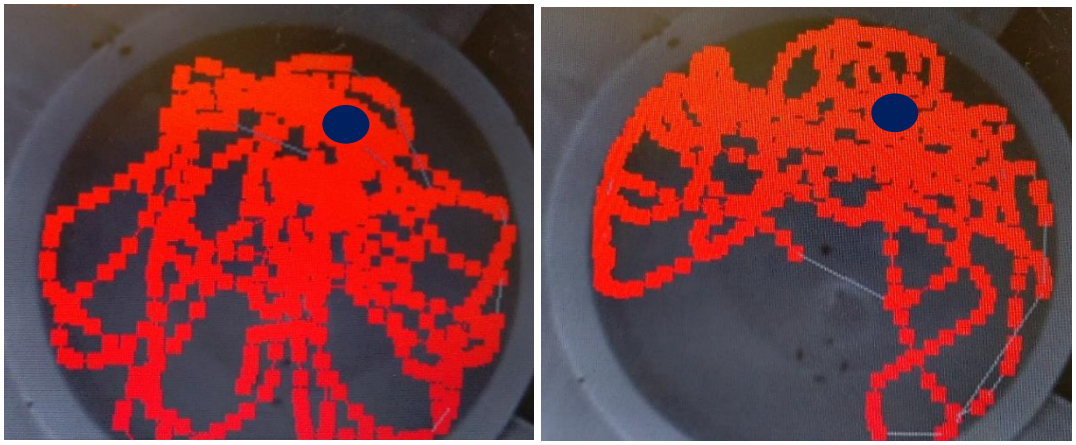

Figure B.11: Tracked behaviour of the rat in the Morris water maze using Ethovision software.

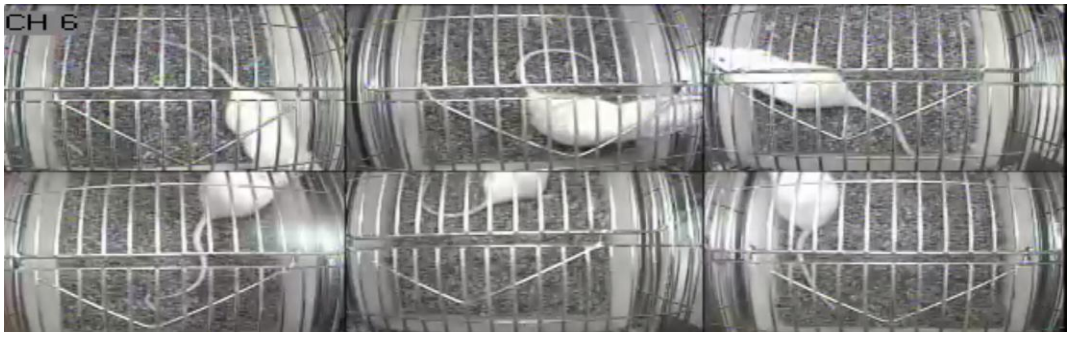

Figure B.12: Home cage activity apparatus.

Figure B.13: Tracked behaviour of the rat in the home cage activity using Ethovision software.