Thermoregulatory, behavioural and neurochemical effects of 3,4-methylenedioxymethamphetamine (MDMA) and related stimulant drugs

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is an amphetamine derivative widely used in rave party and club scenes. In some users, MDMA causes fatalities, most often due to acute hyperthermia which leads eventually to multi-organ failure. Other structurally related drugs, including methamphetamine and para-methoxyamphetamine (PMA), as well as structurally unrelated cocaine, have also been associated with death due to hyperthermia, and are also often taken with or instead of MDMA. Harm minimisation advice to prevent this acute hyperthermia depends on appropriate thermoregulatory behaviour by drug users, an aspect of thermoregulation which had not been studied with respect to MDMA previously.

The purpose of this thesis was to use a novel behavioural thermoregulation model in rats to investigate the effects of MDMA and other stimulant drugs on behavioural thermoregulation and related physiological parameters, as well as investigating residual neurochemical changes caused by these substances.

The behavioural thermoregulation model used throughout most of this thesis involved rats being administered a drug, immediately prior to being confined to a set ambient temperature (30 ± 1° or 21.5 ± 1.5ºC) for 30 minutes. Rats were then immediately allowed access to a thermally graded runway (11-41ºC) where they were able to choose their preferred temperature for a further 4 hours. The final study consisted of giving rats a drug in their home cages at an elevated ambient temperature.

Firstly, a dose-response study was conducted using MDMA, PMA, methamphetamine and cocaine. All drugs lead to a dose dependent increase in core temperature at high ambient temperature, and this led to animals seeking the cool end of the runway after MDMA, methamphetamine and cocaine administration, but not after PMA. Methamphetamine was the most potent drug at increasing core temperature, followed by MDMA and PMA, then

cocaine as the least potent, however, MDMA and PMA showed steeper slopes on the dose-response curves than methamphetamine and cocaine.

The second study consisted of rats receiving MDMA at 30 or 21.5ºC for three consecutive days a week for one week or 6 weeks before being tested in the thermal gradient. The main findings of this study were that heart rate (HR) response to MDMA progressively decreased with repeated dosing over 6 weeks at both ambient temperatures, and that there was a difference in core temperature between rats treated for 6 weeks compared to 1 week when they were in the thermal gradient.

The third study looked at the effects of MDMA in an animal model of depression, the Flinders Sensitive Line (FSL) rat. We showed that FSL rats were much more sensitive to the effects of MDMA at a high ambient temperature compared to Sprague-Dawley controls, however there were limited differences in behaviour in the thermal gradient between the strains. Pharmacokinetic analysis showed that there was no difference in blood or brain concentrations of MDMA, or its metabolite 3,4-methylenedioxyamphetamine (MDA) which could have explained the different responses. These concentrations also showed that the dosing regimens used throughout this thesis led to similar plasma concentration as those reported in human users.

The final study was a pilot study done to see if proteomics could be a useful method to investigate the effects of MDMA and other stimulants on the brain after administration at a high ambient temperature. Rats were administered MDMA, methamphetamine or a combination, and several changes in protein expression were found. These were mostly evident in rats treated with MDMA which was in contrast to the effects on neurotransmitter concentration and acute hyperthermia, which was only seen in rats treated with MDMA and methamphetamine together.

Three of the four results chapters in this thesis have been published or have been accepted for publication, while the fourth has been prepared as a manuscript ready for publication.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Emily Jaehne and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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A be havioural, ne urochemical a nd pr oteomic a nalysis a fter t reatment w ith 3,4 - methylenedioxymethamphetamine and methamphetamine.

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Signed   Date   05/02/10

Dr Penno c onducted s tatistical a nalysis of pr oteomic r esults a nd c ontributed t o t he interpretation of t he da ta c ollected a nd pr eparation of t he m anuscript, i ncluding w riting much of the proteomics sections of the Methods and Results.

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Abbreviations, prefixes and symbols

MDMA   3,4-methylenedioxymethamphetamine

PMA   para-methoxyamphetamine

MDA   methylenedioxyamphetamine

MDEA   methylenedioxyethamphetamine

5HT   5-hydroxytryptamine/serotonin

5HTT   serotonin transporter

DA   dopamine

CNS   central nervous system

MAO   monoamine oxidase

HHMA/DHMA/N-Me-α-MeDA   3,4-dihydroxymethamphetamine

HHA/DHA/α-MeDA   3,4-dihydroxyamphetamine

6-OH-MDMA 2-hydroxy-4,5-methylenedioxymethamphetamine

COMT   catechol-O-methyl transferase

HMMA   4-hydroxy-3-methoxymethamphetamine

HMA   4-hydroxy-3-methoxyamphetamine

CYP450   cytochrome P450

LMA   locomotor activity

NA   noradrenaline/norepinephrine

POAH   preoptic anterior hypothalamus

LPS   lipopolysaccharide

T_C core temperature

AMPT α-methyl-p-tyrsine

DOI   ($\pm$)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopronane

8-OH-DPAT   8-hydroxy-2-(di-N-proylamino)tetralin

5HIAA   5-hydroxyindole acetic acid

HPLC	high performance liquid chromatography
SD	Sprague-Dawley
T_P	preferred temperature
SERT	serotonin reuptake transporter
DOPAC	dihydroxyphenyl acetic acid
FSL	Flinders Sensitive Line
HR	heart rate
ED_{50}	dose of 50% effective response
MAP	mean arterial pressure
T_A	ambient temperature
2-DE	2-dimensional electrophoresis
MS	mass spectrometry
METH	methamphetamine
ACON	aconitate hydratase
UB2V1	ubiquitin-conjugating enzyme E2 variant 1
MEK1	mitogen-activated protein kinase 1
GSTO1	glutathione transferase omega-1
SSRI	selective serotonin reuptake inhibitor
DAT	dopamine transporter

1.   Research Background

3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is an amphetamine derivative widely used in rave party and club scenes. This drug is thought to be less harmful than perceived 'hard' drugs such as heroin and cocaine, due to its positive mood enhancing and stimulant effects and supposed lack of major acute side effects. However, in some users, MDMA causes fatalities, most often due to acute hyperthermia which leads eventually to multi-organ failure. Other structurally related drugs which have also been associated with death due to hyperthermia are also often taken with or instead of MDMA. These ingestions are either intentional or unknowingly in ecstasy tablets containing the drugs in addition to MDMA. Two of the main compounds in this category are methamphetamine and para-methoxyamphetamine (PMA) (Byard *et al*, 1998; Irvine *et al*, 2006).

Although it is clear hyperthermia may be dangerous for stimulant users, there has been difficulty in predicting when these adverse effects will occur. Wide ranges of plasma concentrations of MDMA have been reported in fatal cases (Caldicott *et al*, 2003; Gowing *et al*, 2002), and these concentrations often overlap with those associated with only a minor change in core temperature and other physiological effects (Irvine *et al*, 2006). There also appear to be many contributing factors to hyperthermia including environment, poly drug use and behaviour. The full extent of the combined role each factor plays has not yet been fully elucidated. Although extrapolating results obtained from animal studies must be made with caution (Easton and Marsden, 2006), animal models are an essential tool in establishing possible problems associated with particular drugs (de la Torre and Farre, 2004; Easton and Marsden, 2006). For example, animal studies have shown that changes in body temperature induced by stimulant drugs are dependent on ambient temperature (Jaehne *et al*, 2005; Lomax and Daniel, 1990; Stanley *et al*, 2007; Xie *et al*, 2000), which may be important for human users taking these drugs in warm clubs. Two of

the deficiencies of previous animal studies at the time of starting this thesis are firstly, that the behavioural aspect of thermoregulation had not been investigated, and secondly, that many of the studies used rectal or ear temperature measurements. Measuring temperature in these ways requires handling of the animals, which can lead to stress induced increases in core temperature (Gordon, 1990). Some studies have also used mouse models, which are not an appropriate model for MDMA research relevant to human health, as their neurochemical and behavioural responses to MDMA are distinctly different from rats, non-human primates and humans, which are all similar (Lyles and Cadet, 2003; McCann *et al*, 1998; Mechan *et al*, 2006). We therefore decided to look at behavioural thermoregulation, using surgically implanted telemetry devices to measure core temperature in rat models of MDMA use.

The primary aim of this project was to investigate the effects of MDMA on thermoregulation, with a focus on behavioural thermoregulation. The secondary aim was to investigate the longer term effects on neurochemical function associated with MDMA. In some cases, the results were compared to related stimulant drugs, most importantly methamphetamine and PMA. The approach involved *in vivo* investigation of core temperature under different conditions, using a biotelemetry system, and a variety of methods to study the effect of treatments on the brain.

1.1.   Historical Origins of MDMA

MDMA was first synthesised by the German pharmaceutical company Merck in 1912 as part of a new pathway leading to haemostatic substances (Freudenmann *et al*, 2006), but not for any use in itself. It was then studied by the USA army in the 1950's, along with other mescaline analogs in five animal models, although this work was not published until 1973 (Hardman *et al*, 1973). Soon after this the time the first reports of the psychoactive properties of the compound in human users were published (Anderson *et al*, 1978). In the

1980's MDMA was tested as a psychotherapeutic aid (Greer and Strassman, 1985; Greer and Tolbert, 1986; Grinspoon and Bakalar, 1986), and although this was abandoned due to the risk of abuse, it has been looked at again more recently (Greer and Tolbert, 1998; Sessa, 2007). MDMA started to be used recreationally by small numbers of people in the 1970's. After it was classified as a Schedule 1 drug in 1985, it started to become more popular due to the related publicity in the USA and Europe (Parrott, 2001). It then continued to increase in popularity during the 1980's and 1990's, especially in "rave" or "techno" dance parties (Green *et al*, 2003), and as reported above, has continued to grow in popularity around the world as the drug "ecstasy".
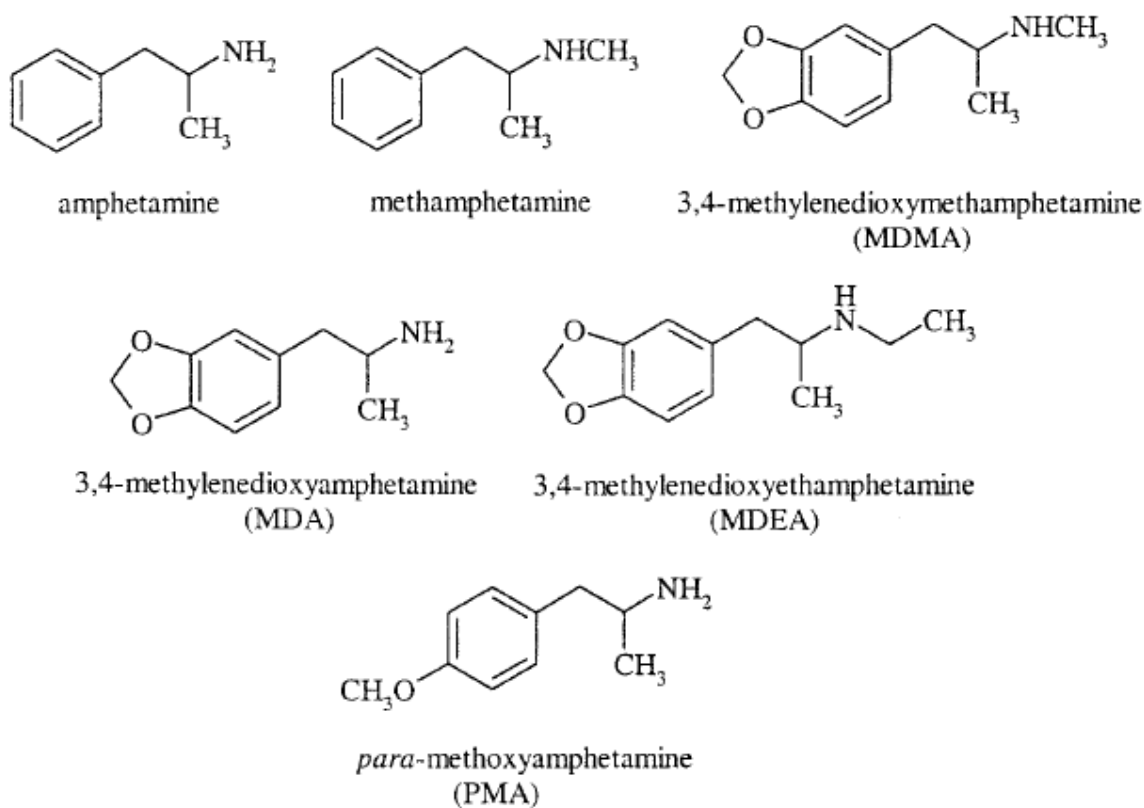
Figure 1. Chemical structure of MDMA and related amphetamine derivatives.

The term ecstasy most commonly refers to MDMA, but is often a mixture of different amphetamine derivatives and other drugs. While the majority of tablets contain MDMA, they also often contain other related chemicals such as 3,4-methylenedioxyamphetamine (MDA) or 3,4-methylenedioxyethamphetamine (MDEA), PMA, methamphetamine,

amphetamine (see Figure 1 for chemical structures), as well as other types of drugs like ketamine, ephedrine, salicylates and over the counter painkillers (Cole *et al*, 2002).

1.2. Prevalence of MDMA Use

Several reports state that the use of MDMA has recently risen around the world, with increases of over 60% in use of 'ecstasy' by young people in all Western regions from the early 1990's until 2002 (United Nations, 2003). Use is also either still rising or staying constant in many parts of the world (United Nations, 2008). Australia has the highest per capita use of 'ecstasy' in the world. The 2007 National Drug Strategy Household Survey showed that 3.5% of the population aged 14 years or older had used the drug in the last 12 months (Australian Institute of Health and Welfare, 2008a). This equates to 4.4% of 15-64 years olds, which is more than double the use in almost every other country in the world (e.g. Czech Republic 3.5; New Zealand 2.6; England and Wales 1.8; Northern Ireland 1.8; Canada 1.3; USA 1.0%) (United Nations, 2008). 'Ecstasy' is also the highest used illicit drug behind cannabis in Australia, with 9.1% of people aged 14 years or older using cannabis, 2.5% using meth/amphetamine, 1.6% using cocaine and 0.2% using heroin in the previous 12 months (Australian Institute of Health and Welfare, 2008b).

1.3. Effects in Human Users

1.3.1. Desirable

The desirable effects of MDMA, which lead to the use of ecstasy, are generally psychological. They include feelings such as euphoria, reduction of negative thoughts, increased energy, happiness, friendliness, calmness, relaxation and heightened perception of sound, colour and touch (Baylen and Rosenberg, 2006; Davison and Parrott, 1997; Green *et al*, 2003).

Its attractiveness as a recreational drug is also dependent on the perception that it is a safe drug. It is primarily used in pill form and thus not associated with unpleasant aspects of intravenous drug use. It is also a drug with low potential for dependence and addiction although this has been challenged by some authors (Jansen, 1999). MDMA is also not associated with aggressive behaviours which is often the case with other recreational drugs. In combination, these perceptions probably explain its general popularity and its acceptance by young females who are generally more risk aversive when compared to young males.

1.3.2.   Acute adverse effects

There are also many adverse effects associated with ecstasy use. Acute adverse effects associated with MDMA use include motor and muscular problems, such as hyperactivity, muscle aches and tension, and bruxism and jaw clenching, as well as others such as elevated blood pressure and heart rate, nausea, chills, sweating, confusion and hyperthermia (Green *et al*, 2003; Lyles and Cadet, 2003; McCann *et al*, 1996). Although the epidemiological evidence indicates the incidence of major adverse effects are low (Byard *et al*, 1998), the events are unpredictable and can lead to death or morbidity (Gowing *et al*, 2002; Williamson *et al*, 1997). Hyperthermia is one of these major effects, which can lead to death due to cardiac arrhythmias, acute renal failure, rhabdomyolysis and disseminated intravascular coagulation (Lyles and Cadet, 2003; Screaton *et al*, 1992). The clinical picture of an 'ecstasy overdose' victim is very similar to that described as 'serotonin syndrome'. The serotonin syndrome, believed to be caused by an excess of synaptic serotonin (5-hydroxytryptamine; 5-HT), is usually associated with inappropriate administration of therapeutic drugs which affect the 5HT system (Sun-Edelstein *et al*, 2008). The associated symptoms include hyperactivity, confusion, agitation, jaw clenching, hyperreflexia, hyperthermia, tachycardia, shivering, ocular oscillations, tremor as well as

others (Parrott, 2002). Some of these symptoms such as hyperactivity, confusion and jaw clenching are considered normal effects of ecstasy by users, but some users develop the complications mentioned above. Unfortunately, we are still unaware of why this occurs in some situations and not others, and why it is not a more predictable dose dependent phenomenon as animal studies would suggest.

1.3.3. Long term adverse effects

There are also longer term problems associated with the use of MDMA. Users report adverse feelings such as lethargy, moodiness, irritability, insomnia, paranoia and depression in the days after use of MDMA (Davison and Parrott, 1997; Green *et al*, 2003). It has also been suggested that MDMA use could lead to the development of depression, as long term depletion of serotonin in the brain has been shown in both humans and animals (Malberg and Seiden, 1998; McCann *et al*, 1998; Wang *et al*, 2004). For example, nuclear imaging studies have shown consistently that ecstasy users have reduced serotonin transporter (5HTT) levels, a possible indicator for loss of 5HT neurons (McCann *et al*, 1998; 2005; Reneman *et al*, 2002a; 2002b; 2002c). However, several studies show evidence that depression may actually precede MDMA use in many users (Guillot and Greenway, 2006; Lieb *et al*, 2002; Soar *et al*, 2001). There is evidence for other long term cognitive problems associated with ecstasy use, although high rates of poly drug use makes it difficult to interpret some results. Several studies have attempted to account for this in different ways. Halpern *et al* (2004) found a group of MDMA users and non-users with low exposure to other types of drugs including alcohol and tobacco. They were able to show that heavy MDMA users displayed deficits in mental processing speed and impulsivity compared to non-users. Other groups have compared ecstasy users to cannabis users, to control for the high cannabis use in the drug using population. Quednow *et al* (2006a; 2006b) found that MDMA users also showed higher impulsivity and lower

decision making performance compared to cannabis users and non-drug users (Quednow *et al*, 2006b), as well as memory deficits compared to the same control groups (Quednow *et al*, 2006a).

1.4.   Mechanism of Action

The mechanism of action of MDMA is not fully understood, but it is known that it has a stimulant effect on the brain. It works to acutely increase extracellular concentrations of neurotransmitters such as 5-HT and dopamine (DA) in the central nervous system (CNS), as well as acting directly on postsynaptic receptors for these neurotransmitters (Cole and Sumnall, 2003). MDMA increases the release of neurotransmitters in several ways (Figure 2). It acts as a substrate for vesicular and plasma membrane reuptake transporters, reversing their action so that high amounts of neurotransmitter are released into the synaptic cleft (Cole and Sumnall, 2003). It also acts to inhibit monoamine oxidase (MAO), which is the enzyme involved in breaking down monoamines such as 5HT and DA, inside and outside the cell, hence resulting in higher amounts of them being available to bind to postsynaptic receptors (Leonardi and Azmitia, 1994). MDMA administration also leads to increased release of hormones such as cortisol (Connor *et al*, 1998; Mas *et al*, 1999), oxytocin (Thompson *et al*, 2007; Wolff *et al*, 2006) and ghrelin (Kobeissy *et al*, 2008) and decreases in leptin, growth hormone and neuropeptide-Y (Kobeissy *et al*, 2008) which may be involved in some of the psychological effects of MDMA.

The affinity of MDMA to bind to the reuptake transporters and postsynaptic receptors of the different neurotransmitters varies, as with all drugs (Battaglia *et al*, 1988; Steele *et al*, 1987). Table 1 summarises these affinities. It is important to note that MDMA has the highest affinity for the 5HT uptake transporter, so a high proportion of its effects are likely to come from this interaction. Battaglia *et al* (1988) have also shown that MDMA and MDA have similar affinity for most receptors, while amphetamine and

methamphetamine have lesser affinity for 5HT and muscarinic receptors, but greater affinity for adrenoceptors (Table 2).
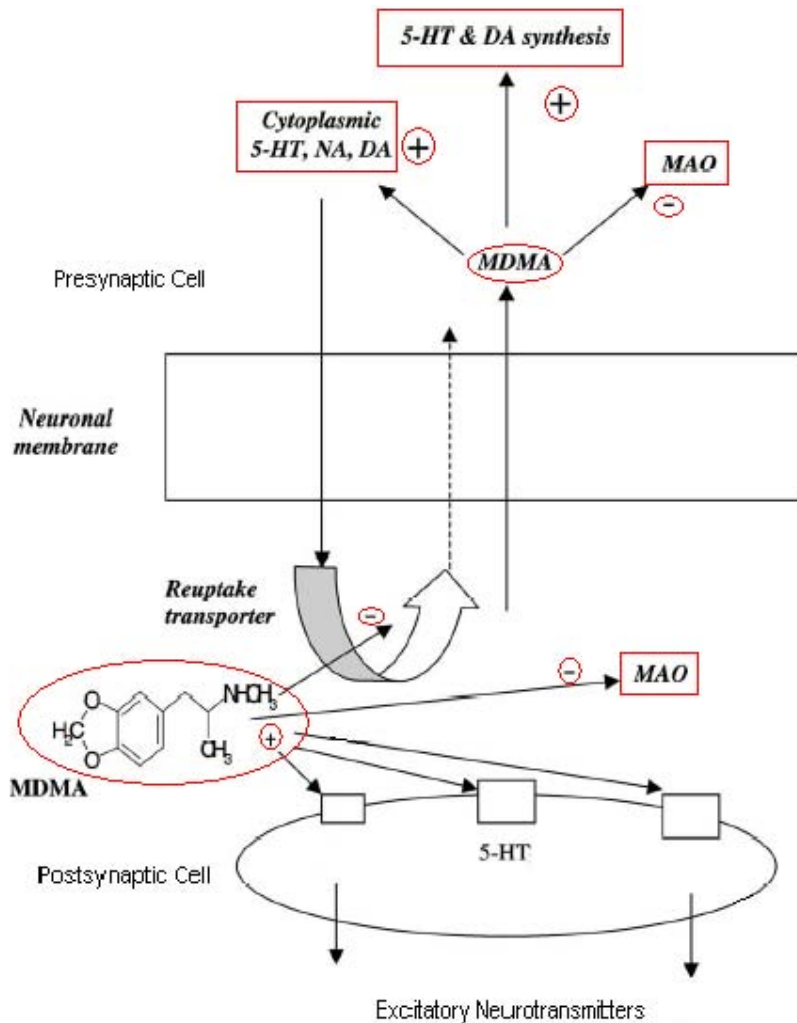
Figure 2. Main pharmacological effects of MDMA. See text for details. Derived from Cole & Sumnall (2003)

The regions of the brain where MDMA is thought to have its greatest effects are consistent with the location of receptors MDMA has high affinity for, and with the reported and observed effects of the drug. Several rat studies have looked at the expression of Fos, a marker of neural activation and shown increased expression in areas of the brain with high amounts of 5HT and DA neurons, as well as areas known to be involved in thermoregulation, social and emotional behaviours, balance and coordination and

locomotor activity (Colussi-Mas and Schenk, 2008; Erdtmann-Vourliotis *et al*, 1999; Hargreaves *et al*, 2007; Stephenson *et al*, 1999). These areas include the median preoptic nucleus and regions of the cerebellum, amygdala, striatum and hypothalamus, as well as others. Measurement of blood flow in different parts of the brain using PET scanning in pigs and humans has also shown changes in activation of various areas of the brain which could explain some effects of MDMA (Gamma *et al*, 2000; Rosa-Neto *et al*, 2004).

Brain recognition site	Affinity Ki (µM)
Serotonin Uptake Transporter	0.61 ± 0.05
Noradrenalin Uptake Transporter	15.7 ± 1.7
Dopamine Uptake Transporter	24.4 ± 1.9
α_1 Adrenoceptor	18.4 ± 1.2
α_2 Adrenoceptor	3.6 ± 0.8
β Adrenoceptor	19.2 ± 2.1
D1 Dopamine Receptor	148 ± 14
D2 Dopamine Receptor	95 ± 15
$5HT_1$ Serotonin Receptor	23 ± 1.5
$5HT_2$ Serotonin Receptor	5.1 ± 0.3
M1 Muscarinic Receptor	5.8 ± 0.3
M2 Muscarinic Receptor	15.1 ± 0.1
H1 Histamine Receptor	5.7 ± 2.4

Table 1. Affinity of MDMA for major recognition sites in the brain. Derived from Battaglia *et al* (1988)

Drug	5HT Uptake	$5HT_2$ Receptor	α_2 Adrenoceptor	M1 Receptor
MDMA	1.0	1.0	1.0	1.0
MDA	1.8	0.5	0.5	1.4
MDE	0.4	3.5	3.3	1.8
Amphetamine	4.8	2.6	0.09	4.8
Methamphetamine	3.4	2.4	0.61	3.6

Table 2. Relative potencies of amphetamine derivatives at selected receptors in the brain. Comparisons of affinities with respect to MDMA at these sites. Derived from Battaglia *et al* (1988)

1.5. Metabolism

MDMA is metabolised into many different compounds (Figure 3). Initially, in the rat, it is N-demethylated to MDA, O-demethylated to 3,4-dihydroxymethamphetamine (HHMA/DHMA/N-Me-α-MeDA) and ring hydroxylated to 2-hydroxy-4,5-methylenedioxymethamphetamine (6-OH-MDMA). MDA, which as discussed previously

has similar pharmacological activity to MDMA, is further O-demethylated to 3,4-dihydroxyamphetamine (HHA/DHA/α-MeDA). HHMA and HHA can be metabolised into many other products, some of which are neurotoxic and thought to contribute to the long term neurochemical effects of MDMA (Capela *et al*, 2009; de la Torre and Farre, 2004; Green *et al*, 2003). The corresponding quinones of HHMA and HHA conjugate with glutathione and the resulting thioether conjugates have been shown to be neurotoxic when applied directly to the brains of rats, where MDMA and MDA have been shown to not have this effect (Monks *et al*, 2004). Examples of other metabolites of MDMA which have been shown to neurotoxic *in vivo* are shown in Table 3. HHMA and HHA are otherwise deactivated via catechol-O-methyl transferase (COMT) to 4-hydroxy-3methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA) respectively. It is therefore clear that the pharmacology of MDMA is very complicated, and both active and neurotoxic metabolites are likely to contribute to the overall effects of the drug (de la Torre and Farre, 2004; Green *et al*, 2003).
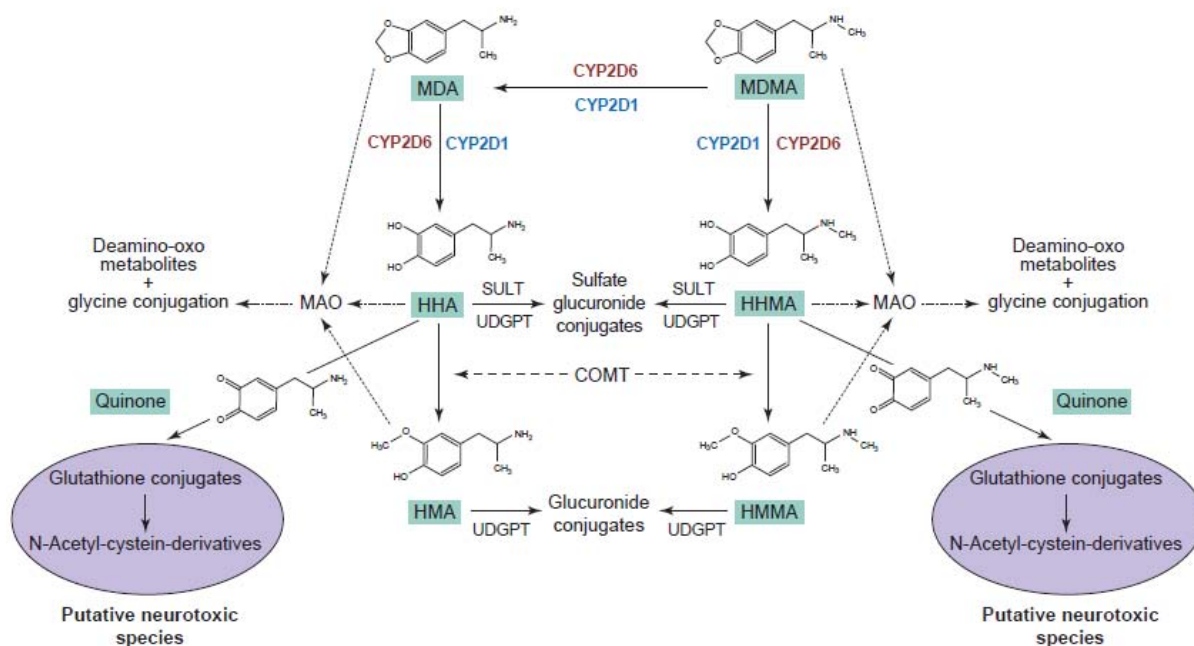
Figure 3. Some of the pathways of MDMA metabolism in rats and humans. The main CYP450 enzymes involved in rats are highlighted in blue, and enzymes involved in humans are highlighted in red. Derived from de la Torre and Farre (2004).

MDMA Metabolite	Neurotoxicity *in vivo*
HHA	ICV administration was not neurotoxic; s.c. administration produced long term 5HT neurotoxicity in rats
HHMA	i.p. administration was not neurotoxic; ICV administration produced DA neurotoxicity in mice
HMA	s.c. administration produced long term 5HT neurotoxicity in rats
5-(GSH)-α-MeDA	ICV administration did not induce long-term neurotoxicity; direct intrastriatal or intracortical administration caused long-term 5HT neurotoxicity in rats
5-(NAC)-α-MeDA	ICV administration did not induce long-term neurotoxicity; direct intrastriatal or intracortical administration caused long-term 5HT neurotoxicity in rats
5-(NAC)-*N*-Me-α-MeDA	Direct intrastriatal administration caused long-term 5HT neurotoxicity in rats
2,5-(GSH)-α-MeDA	ICV or direct intrastriatal or intracortical administration caused long-term 5HT neurotoxicity in rats

Table 3. Examples of neurotoxicity of MDMA metabolites. Derived from Capela *et al* (2009).

It should also be noted that the metabolism of MDMA differs between humans and rats. In humans O-demethylation of MDMA to HHMA is the main pathway, whereas in rats it is N-demethylation to MDA. This may affect the acute effects of the drug as MDA is active and HHMA is not (Chu *et al*, 1996; de la Torre and Farre, 2004). There are also differences in the cytochrome P450 (CYP450) enzymes involved in metabolism between species. In rats CYP2D1 is the main enzyme involved and in humans it is the homologous but not functionally identical to the CYP2D6 enzyme. MDMA inhibits the CYP2D6 enzyme in humans, whereas it does not do this to the rat CYP2D1 enzyme, which contributes to differences in the rate of metabolism between species (de la Torre and Farre, 2004). One ecstasy pill in a human user will inhibit CYP2D6 metabolism for at least 24 hours. This results in high sustained concentrations of the parent drug in individuals who take multiple doses on one occasion. The CYP2D6 enzyme is also highly polymorphic in humans, so that poor, extensive and normal metabolisers may have different acute responses to the drug, as well as different neurotoxic potential as different amounts of neurotoxic metabolites could be formed. Female Dark Agouti rats are CYP2D isoenzyme

deficient and are an animal model of CYP2D6 poor metabolisers. These rats show a greater hyperthermic effect after MDMA administration, and possibly a lower amount of neurotoxic damage to the brain, although contrasting results have been shown (Chu *et al*, 1996; Colado *et al*, 1995; Malpass *et al*, 1999). However, because of the inhibition of this enzyme by MDMA in humans, which effectively makes all humans phenotypically poor metabolisers regardless of their CYP2D6 genotype, CYP polymorphism may not be very important in explaining variations in drug response.

1.6.   Optical Isomers of MDMA

The percentage of each of the two optical isomers, or enantiomers, of MDMA present in 'ecstasy' tablets used by humans is unknown, although it is generally presumed to be racemic. However, there are important differences in the effects of the two enantiomers which should be discussed. The S(+) isomer of MDMA, as well as of MDA and amphetamine, is more potent at binding the 5HT and DA transporters, leading to greater release of these neurotransmitters compared to the R(-) isomer (Steele *et al*, 1987), while the R(-) isomer has higher affinity for $5HT_1$ and $5HT_2$ receptors (Lyon *et al*, 1986). There are also different effects when only a single isomer of MDMA is administered to rats. Administration of S(+)-MDMA to rats leads to higher stereotyped behaviours such as head weaving, back pedalling and turning than R(-)-MDMA (Hiramatsu *et al*, 1989). S(+)-MDMA was more potent than S(+)-MDMA in eliciting some stereotyped behaviours, particularly wet-dog shakes (Hiramatsu *et al*, 1989), which may also contribute to the overall effects after administration of MDMA. This result is similar in mice, where it has been shown that both racemic and S(+)-MDMA administration leads to higher locomotor activity (LMA) than R(-)-MDMA (Fantegrossi *et al*, 2003). This paper also showed that only racemic and S(+)-MDMA produce hyperthermia, while R(-)-MDMA does not, and that R(-)-MDMA also produces less lethality than racemic or S(+)-MDMA at the same

dose (Fantegrossi *et al*, 2003). However, many of the effects of MDMA in mice are very different to rats and humans (Green *et al*, 2003), so any results reported in mice may not translate to other species.

There are also differences in the way each of the isomers are metabolised which may contribute to the different effects when only one enantiomer is administered. When S(+)-MDMA is administered via i.v. or subcutaneous injection, levels of MDA formed are approximately three times higher than when R(-)-MDMA is administered, while levels of MDMA are the same (Cho *et al*, 1990; Hiramatsu *et al*, 1991). However, when racemic MDMA is administered intravenously (i.v.) (20 mg/kg) the percentage of the dose excreted as R(-)-MDMA is 20 ± 10%, compared to 12 ± 6% for S(+)-MDMA. 3 ± 1% of the dose is excreted as R(-)-MDA and 6 ± 2% is excreted as S(+)-MDA (Fitzgerald *et al*, 1990).

1.7. Thermoregulation

Normal body temperature control in all mammals involves both autonomic and behavioural responses. Metabolic processes involved in normal cellular function in mammals produce heat which leads to a constant core body temperature being maintained (Rusyniak and Sprague, 2005). When there is a change in core body temperature autonomic responses are initiated, mostly via signals from the hypothalamus. Autonomic responses to heat include sweating and cutaneous vasodilation, while responses to cold include shivering and vasoconstriction (Roberts and Martin, 1977; Rusyniak and Sprague, 2005; Sessler, 1997). Behavioural thermoregulation is the most effective response, as thermal discomfort leads to responses such as changing clothes in humans or moving to a more comfortable area in both humans and other mammals, which minimises autonomic thermoregulatory strain and saves energy (Attia, 1984; Sessler, 1997). The main neurotransmitters involved in thermoregulation are 5-HT (Rothwell, 1994), DA (Rusyniak and Sprague, 2005) and noradrenaline (NA) (Mallick *et al*, 2002), all of which are effected

greatly by the presence of stimulant drugs. Therefore, drugs such as MDMA, and other stimulants which directly act on these neurotransmitter systems, would be expected to disrupt many aspects of thermoregulation.

There are several ways in which behavioural thermoregulation can be studied in rats. Humphreys *et al* (1976) looked at the effect of different ambient temperatures, following injections of anaesthetic into brain sites including the preoptic anterior hypothalamus (POAH), on rectal temperature and behavioural thermoregulation. When rats pressed a lever, a lamp would turn off, and an exhaust fan would draw air in from the surrounding room, hence cooling the chamber. Injections of sodium pentobarbital (Nembutal) into the POAH decreased time spent escaping heat. The body temperature of these animals was also increased in neutral (23°C) and cold (10°C) environments, and failed to show a normal decrease in hot (34°C) environments, indicating the importance of this brain region in both autonomic and behavioural control of body temperature.

Another method used to demonstrate effects on behavioural thermoregulation is that of thermal gradients. Florez-Duquet *et al* (2001) have looked at fever responses in young and old rats, using telemetry implants to determine body temperature. Their thermal gradient consisted of an aluminium floor and wall, with heating tape on one end and the other end bathed in circulating chilled ethylene glycol, resulting in a floor temperature gradient of 10-40°C. Lipopolysaccharide (LPS) was injected into rats placed in their home cages for three hours, after a three-hour habituation period in the thermal gradient. Body temperatures of young animals increased during this time, while no change was shown in old rats. That is until they were placed back in the thermal gradient and allowed to behaviourally thermoregulate. Older rats chose to sit in areas of higher temperature than young rats, and displayed a higher fever.

Other studies have also used similar methods to look at behavioural thermoregulation. Gordon (1987) looked at the relationship between preferred ambient temperature and

autonomic thermoregulation in rats. He measured ambient temperature in the gradient above the floor and found rats of different strains chose ambient temperatures lower than those that resulted in a minimal metabolic rate and constant body temperature. In another experiment Gordon *et al* (2000) put mice on a gradient consisting of a floor of copper bars with the ambient temperature being set at different temperatures. For the first hour mice ran up and down the runway with no preferred temperature, but during the second hour at ambient temperatures of 25, 27.5 and 40°C mice predominantly chose floor temperatures around 25°C while at 30, 32.5 and 35°C they chose higher temperatures of around 35°C. These experiments show that rodents will behaviourally thermoregulate appropriately in this type of apparatus if there is a requirement to do so and they have the ability and motivation.

1.8. Effect of Stimulant Drugs on Thermoregulation

1.8.1. MDMA

Studies in our laboratory have measured both autonomic and behavioural effects of MDMA and have shown that MDMA has different effects on core body temperature (T_C) of rats depending on ambient temperature. This has also been shown by other laboratories in various strains of rats. Dafters and Lynch (1998) reported that MDMA doses of 10 or 15 mg/kg resulted in significant increases of up to 2.32°C at an ambient temperature of 22°C, and decreases of 2.75°C at an ambient temperature of 17°C in female Wistar rats. Malberg and Sieden (1998) used higher doses of 20 and 40 mg/kg and showed hyperthermia compared with saline controls at ambient temperatures of 28 and 30°C, and hypothermia at 20 and 22°C in male Holtzman rats.

The effect of MDMA on thermoregulation can change when it is given repeatedly over a longer period of time. Like many drugs, animals demonstrate tolerance or sensitization to some or all effects of the drug. However, the results of studies looking at

these effects are contrasting. Green *et al* (2004b) showed that a prior neurotoxic dose of 12.5 mg/kg MDMA resulted in a larger hyperthermic response to a subsequent 5 mg/kg dose over 1 week later. O'Shea *et al* (1998) gave MDMA (4mg/kg) twice weekly for 8 weeks and showed a constant hyperthermic response at normal ambient temperature, i.e. no tolerance or sensitization to the effects of MDMA. Clemens *et al* (2007) gave MDMA (8mg/kg) at an elevated ambient temperature (28°C) once a week for 16 weeks and found that tolerance to the hyperthermic effects of MDMA developed after the 1st week. A major difference between these studies is the ambient temperature that the rats were treated at, which as mentioned above, can influence the effect MDMA has on T_C in rats.

There is debate over which neurotransmitter systems are most important in the effects of MDMA on core temperature. Several studies have used 5-HT and DA uptake inhibitors and receptor antagonists to prevent MDMA-induced hyperthermia but have shown variable results. Mechan *et al* (2002) used a 12.5 mg/kg dose of MDMA at 20 ± 2°C which produced a significant increase in rectal temperature. Pre-treatment with several 5-HT receptor antagonists and uptake inhibitors administered 20 minutes before MDMA had no effect on hyperthermia. The only drug they found which prevented MDMA-induced hyperthermia was the DA D_1 receptor antagonist SCH 23390 (0.3-2 mg/kg). In contrast, Malberg *et al* (1996) found pre-treatment with the 5-HT$_{2A}$ antagonist ketanserin (6 mg/kg) and the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine (AMPT) (75 mg/kg) prevented the hyperthermic effect of a 40 mg/kg dose of MDMA at 22°C.

Other studies have shown activation of postsynaptic 5-HT$_{2A}$ receptors using (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) causes hyperthermia through cutaneous vasoconstriction in both rabbits and rats (Blessing and Seaman, 2003), while inhibitory presynaptic 5-HT$_{1A}$ receptor activation with 8-hydroxy-2-(di-N-propylamino)tetralin (8-OH-DPAT) leads to vasodilation and a fall in body temperature in rabbits (Ootsuka and Blessing, 2003). The same lab subsequently showed that MDMA also works via a

vasoactive mechanism (Blessing *et al*, 2003). Rats were given MDMA (10 mg/kg) at an ambient temperature of 26-28°C. 90 minutes later, their body temperature had risen around 1.5-2°C and their tail artery blood flow had decreased by 50% (indicating vasoconstriction). Clozapine, an atypical antipsychotic agent with antagonist action on DA D_4 receptors, as well as several 5-HT and muscarinic receptors, reversed both effects of MDMA. It actually increased the blood flow to a higher level than prior to any treatment suggesting possible involvement of several neurotransmitter systems. Overall, these results suggest both 5-HT and DA neurotransmitter systems may be involved in the effects of MDMA on thermoregulation.

1.8.2. PMA

PMA is a stimulant drug, which is similar in structure (Figure 1) and effect to MDMA. It has been found in some ecstasy tablets, and very rarely used knowingly as a drug in its own right. Case studies suggest that, while it is used to a much lesser extent than MDMA, it is more likely to cause life threatening hyperthermia in human users than MDMA (Byard *et al*, 1998; Caldicott *et al*, 2003; Irvine *et al*, 2006; Ling *et al*, 2001). Our previous work has shown that a 10 mg/kg dose of PMA resulted in the same level of hyperthermia as an equivalent dose of MDMA during a 30 minute confinement to an ambient temperature of 30°C, although subsequent choice of ambient temperature on a thermal gradient was different (Jaehne *et al*, 2005).

In contrast, Freezer *et al* (2005) showed a greater increase in tympanic temperature of 10 mg/kg PMA treated rats compared to the same dose of MDMA after 60 minutes at room temperature. This study also looked at the levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the striatum of the rat using *in vivo* microdialysis and high performance liquid chromatography (HPLC). Rats were injected with 10 mg/kg PMA or MDMA and dialysate samples from the striatum collected every 30 minutes for 5

hours. Both drugs increased 5-HT concentrations in the region while only PMA decreased levels of 5-HIAA, indicating breakdown by MAO was impaired significantly only by PMA. This was reinforced by the results that a MAO inhibitor enhanced the effects of MDMA while not showing any extra effect in PMA treated rats. This shows another difference in the mechanisms of two related compounds which could contribute to any different physiological or behavioural effects that are seen with respect to thermoregulation.

Daws *et al* (2000) actually showed hypothermia after 5-20 mg/kg PMA, greater than that induced by the same doses of MDMA at 20°C. They also conducted chronoamperometry studies to observe differences in the brains of rats after direct administration of PMA or MDMA on the striatum (Daws *et al*, 2000). These studies showed a much greater release of DA and other total neurotransmitters induced by MDMA than PMA in anaesthetised rats. MDMA also had the ability to slow clearance of 5-HT and DA, indicating inhibited reuptake, while PMA only inhibited clearance of 5-HT, showing a selectivity of PMA for 5HT system which may contribute to its thermoregulatory effects.

1.8.3. Methamphetamine

Methamphetamine is another drug of abuse, structurally related to MDMA and PMA, and often used with MDMA, which can also cause hyperthermia. Xie *et al* (2000) looked at the effect of a large dose of methamphetamine (45 mg/kg) in mice at room temperature (22 ± 0.05°C) and another group kept at 6°C from 30 minutes prior to injection until 6 hours after. At 22°C, increases in core temperature up to 39.5°C were reported from a baseline of 38°C, while at 6°C, mice rectal temperature decreased to around 35.5°C from a temperature at the time of injection of about 37°C. Temperatures returned close to baseline by 3 hours into the study. Sprague *et al* (2004) gave 40 mg/kg methamphetamine to Sprague-Dawley (SD) rats at an ambient temperature of 24°C. This dose increased rectal

temperature of the animals by 4°C one hour after administration, which was still apparent an hour later.

Two studies by Clemens and associates have shown different results to the above experiments (2005; Clemens *et al*, 2004). In both, albino Wistar rats were given methamphetamine four times, once every two hours, at an ambient temperature of 28°C. The first study involved male rats receiving 2.5 or 5 mg/kg each injection, and the second using female rats given 4 mg/kg on each occasion. Each regimen showed a trend to slightly increase tympanic temperature but there were no significant differences compared to saline controls in peak temperature achieved. This may be a result of the different dosing regimen used compared to experiments showing an increase.

The effect of methamphetamine on core body temperature seems dependent on DA pathways (Broening *et al*, 2005). Male SD rats were given d-methamphetamine, 10 mg/kg at 24°C, which increased their rectal temperature to over 40°C, and remained elevated after three hours. The D_1 receptor antagonist SCH 23390 significantly prevented this rise after pre-treatment with 0.5 mg/kg, while the D_2 antagonist eticlopride prevented it at 0.05 and 0.5 mg/kg. This protection after both was reversed at an ambient temperature of 33°C suggesting other mechanisms may be involved at high ambient temperatures. There is no evidence of 5-HT being involved in the temperature response to methamphetamine, again suggesting there may be different overall thermoregulatory mechanisms involved with methamphetamine-induced hyperthermia when compared to MDMA and PMA's mechanisms of action.

1.8.4.   Behavioural thermoregulation

One of the desired effects of stimulant drugs such as MDMA is that they can affect perception of sound, colour and touch. They may also therefore affect perception of temperature, which in turn could affect behavioural thermoregulation. There is also

evidence that decision making is impaired in chronic MDMA users (Quednow *et al*, 2006b) and that MDMA can impair decision making acutely in a controlled clinical trial (Vollenweider *et al*, 2005). Any effect on decision making by MDMA or other stimulant compounds could suggest that people under the influence of these drugs may also make inappropriate decisions with respect to keeping their body temperature constant, such as choosing to stay in a warm club instead of a more appropriate location with a lower ambient temperature.

In my honours project (Jaehne *et al*, 2005) we therefore investigated the effect of single doses of MDMA (10 mg/kg), PMA (10 mg/kg) and d-amphetamine (2 mg/kg) on body temperature and behavioural thermoregulation after confinement to different ambient temperatures. This was examined because the behavioural component had not previously been examined with respect to these drugs, in spite of the importance of behaviour in control of normal body temperature and the fact that harm minimisation advice depends on appropriate thermoregulatory behaviour by drug users.

SD rats with telemetry implants measuring T_C, locomotor activity and heart rate were administered the test drug and confined to an ambient temperature of 21, 15 or 30°C for 30 minutes. Rats were then able to choose their preferred position on a thermally graded runway (11 - 41°C) for one hour while temperature preference (T_P) was recorded every 2 minutes.

In the normal environment (21 ± 1°C), T_C of MDMA treated rats fell below that of saline and d-amphetamine treated animals (0.58°C below c.f. 0.8°C above), while rats chose cooler areas of the runway when they had been treated with d-amphetamine than after other treatments. Results were similar after confinement to the cold environment (15 ± 1°C), although the decreased core temperature induced by MDMA was much greater (3.18 ± 0.42°C). Hypothermia in MDMA treated rats reversed and T_C returned to the level

of saline treated controls by 30 minutes into the 60 minute choice period, a result of appropriate thermoregulatory behaviour being employed.

During confinement in the warm environment ($30 \pm 1°C$) T_C of rats treated with either MDMA, d-amphetamine or PMA increased to the same extent. However, there was a difference in choice of ambient temperature after an initial exploratory phase, with PMA treated rats inappropriately choosing the warm end of the runway compared to both MDMA and d-amphetamine treated rats choosing cooler areas, around room temperature. MDMA treated rats may have overcompensated for the increased body temperature though, as their T_C ended up below that of the saline controls after one hour in the runway.

While mostly appropriate behavioural effects were shown, there were differences in both behavioural and thermoregulatory responses observed between drug treatments. This initial study was extended in this thesis to look at a range of doses and drugs that more closely resemble human conditions of drug use.

1.9.   Long Term Residual Effects of Stimulant Drugs (Neurochemical)

1.9.1.   MDMA

There is a link between MDMA administration leading to acute hyperthermia and long term changes in brain concentration of 5HT or related structures in both animals and humans. There are many examples demonstrating this effect, after many different dosing conditions, which will be discussed here. Wang *et al* (2004) gave male SD rats 3 administrations of 7.5 mg/kg MDMA, every 2 hours, and sacrificed them 2 weeks later. They showed tissue concentration of 5HT decreased by 50% in the cortex, hippocampus and caudate compared to controls at this time. Clemens *et al* (2004) have also demonstrated this effect, as long as 7 weeks after giving male albino Wistar rats 4 doses of 5 mg/kg MDMA, each one hour apart, on two consecutive days at $28 \pm 1°C$. Rats treated with MDMA at 10 and 20 mg/kg twice a day for 4 days at 22°C also showed significant

decreases in cortical 5HT and 5HIAA 2 weeks after final administration (Callaghan *et al*, 2006).

These longer term neurochemical effects of MDMA can also be exacerbated when the drug is taken at high ambient temperature, leading to hyperthermia. Malberg & Seiden (1998) gave rats MDMA at a range of ambient temperatures. They showed significant decreases in 5HT concentrations in several brain regions 2 weeks after administration of 40 mg/kg MDMA at 26, 28 and 30ºC, as well as after 20 mg/kg at 30ºC. Broening *et al* (1995) administered 20 or 40 mg/kg MDMA to rats aged 40 or 70 days at various ambient temperatures. Rats showed significant hyperthermia when MDMA was administered at 25 or 33ºC, and large decreases in cortical 5HT measured 1 week later. In contrast, they showed a decrease in T_C after both doses at an ambient temperature of 10ºC, and either no, or much smaller decreases in cortical 5HT concentrations, suggesting a protective effect of low ambient temperature.

High ambient temperature can also increase effects of MDMA in reward areas of the brain, as well as behaviours associated with these areas. MDMA administration leads to greater increases in 5HT and DA in the nucleus accumbens, an area involved in motivational properties of drugs of abuse, at 30ºC compared to 20ºC (O'Shea *et al*, 2005). Increases in MDMA-induced Fos expression, a marker of neural activation have also been reported in this area at high ambient temperatures (Hargreaves *et al*, 2007). Similar increases were also shown in regions associated with social and emotional behaviours and thermoregulation (Hargreaves *et al*, 2007). Supporting these results is another study showing both social interaction and self-administration behaviour by rats is increased when MDMA is given at 30ºC (Cornish *et al*, 2003).

Changes in the brain other than simple decreases in 5HT concentration have also been shown. Hippocampal serotonin reuptake transporter (SERT) binding, synaptosomal 5HT uptake and 5HT concentration were reduced by 15 mg/kg MDMA but not 4 mg/kg,

given to male SD rats twice a day for 4 days (Callaghan *et al*, 2007). Battaglia *et al* (1988) showed dose dependent decreases of 5HT and 5HIAA concentration, as well as 5HT uptake sites, and showed that full recovery had not occurred after 6 months but had after 12. Camarasa *et al* (2006) also showed a decrease in the number of 5HT transporter sites after 10 mg/kg MDMA a day for 4 days, but also showed decreased DA binding in the striatum and down regulation of $5HT_2$ receptors in the cortex. Changes in expression of specific proteins and genes have also been shown, such as changes in mRNA of various glutamate and 5HT receptors in multiple brain regions (Kindlundh-Hogberg *et al*, 2008; 2006).

Studies have also shown similar effects in monkeys (Mechan *et al*, 2006), and there is also evidence showing a link between MDMA administration leading to acute hyperthermia and long term changes in brain concentrations of 5HT or related structures in humans (McCann *et al*, 1998; Volkow *et al*, 2001). For example, brain imaging studies in humans have shown the possibility of long term effects of ecstasy use on human users. Changes were related to the amount of ecstasy used, and included reductions in 5-HT transporter binding (McCann *et al*, 1998; 2005), as well as reductions in $5HT_{2A}$ receptor numbers (Reneman *et al*, 2002c) in recent users. There are problems with interpreting these results, due to poly drug use, as well as being unable to look at brain differences before drug use (Cowan, 2007), but work is continuing in this area. There is also further evidence that 'ecstasy' use may be associated with long term psychobiological problems in human users (Parrott, 2002), suggesting that this may be particularly harmful with increased ambient temperature and perceived body temperature (Parrott *et al*, 2006). Many reports associate MDMA use with clinical depression but a cause/effect relationship has not been established (Guillot and Greenway, 2006; Lieb *et al*, 2002; Soar *et al*, 2001).

1.9.2.   PMA

Little work has been done looking at the effects of PMA on longer term concentrations of neurotransmitters. The studies by Callaghan and associates mentioned above actually compared the effects of MDMA and PMA (Callaghan *et al*, 2006; 2007). They found that PMA decreased cortical 5HT concentrations two weeks after rats were given either 10 or 20 mg/kg twice daily for 4 days, but did not change concentrations of 5HIAA, DA or dihydroxyphenyl acetic acid (DOPAC) (Callaghan *et al*, 2006). A once daily dosing regimen led to decreased paroxetine binding in the cortex 2 weeks after dosing with both 10 and 20 mg/kg, although the decreases were not as great as after 20 mg/kg MDMA (Callaghan *et al*, 2006). Cyanoimipramine binding was also decreased after both 15 mg/kg PMA and MDMA, but not 4 mg/kg, given twice daily for 4 days (Callaghan *et al*, 2007). These results therefore suggest that PMA does less long term damage to serotonergic systems in the brains of rats than MDMA.

1.9.3.   Methamphetamine

Methamphetamine shows a similar ambient temperature dependent neurotoxicity, but unlike MDMA, it causes DA neurotoxicity. Four injections of 5 mg/kg methamphetamine led to a peak core temperature of $38.8 \pm 0.2°C$ at $20°C$, and $40.7 \pm 0.1°C$ at $26°C$. The respective treatments also led to decreases of striatal DA concentrations to 81 and 51% of controls, measured three days after methamphetamine administration (Bowyer *et al*, 1994). In another study the same group of researchers showed similar decreases in 5-HT concentrations at $23°C$, and prevented both neurotoxic effects when the drug was given at $4°C$, using the same doses (Bowyer *et al*, 1992). It has also been shown that methamphetamine given at 4 mg/kg every four hours for a total of four injections can lead to degeneration of cortical neurons and damage to striatal DA and forebrain 5HT terminals (Marshall *et al*, 2007).

Recent studies have demonstrated changes in protein expression in the brains of rats after methamphetamine treatment, using proteomic analysis (Iwazaki *et al*, 2006; 2007; 2008; Li *et al*, 2008; Liao *et al*, 2005). These studies used varying dosing regimens of methamphetamine and showed both up and down regulation of several proteins in the striatum, hippocampus, amygdala and cortex. Specifically, Liao *et al* (2005) showed changes in the striatum 30 minutes after three 10 mg/kg doses of methamphetamine given two hours apart in mice. Li *et al* (2008) showed changes in the striatum, hippocampus and frontal cortex 24 hours after the last of eight 15 mg/kg injections (12 hour intervals). Iwazaki *et al* have shown changes in the striatum and amygdala of rats 4 hours after a single methamphetamine (1 mg/kg) injection (Iwazaki *et al*, 2006; 2008) and 24 hours after a 1 mg/kg dose of MDMA which had followed a dosing regimen 1 week earlier of 1 mg/kg, followed by 10 consecutive days of 4 mg/kg then another day at 1 mg/kg (Iwazaki *et al*, 2007; 2008).

Brain imaging studies in humans have also shown that methamphetamine leads to long term effects on the brain. Changes include reductions in DA transporter in the striatum (Volkow *et al*, 2001) and deficits in markers of both the dopaminergic and serotonergic systems, differences in glucose metabolism, and deficits in gray matter in the cortical and limbic systems (Baicy and London, 2007). These changes are also associated with cognitive problems such as motor slowing and memory impairments (Baicy and London, 2007; Volkow *et al*, 2001).

1.10.   Research Aims

As outlined in the previous section, while much work has been done on the effects of MDMA and other stimulant drugs on physiological aspects of thermoregulation, no one has previously investigated the effects on behavioural thermoregulation. Considering the importance of behaviour in keeping a constant core temperature in all mammals, and the

unpredictability of severe acute hyperthermia in human users, this seems to be a very important aspect to be studied. The main aim of the thesis is therefore to *investigate the acute effects of MDMA and other stimulant drugs on behavioural thermoregulation and related physiological parameters*.

The second aim of the thesis is *to investigate the residual neurochemical changes caused by MDMA and other stimulants*. The longer term effects of MDMA on the brains of rats have been studied extensively, however, different dosing regimens and conditions lead to different outcomes. We have also looked at a novel method of determining the damaging effects of MDMA on the brain in our final paper.

1.10.1. Publication 1: "Pharmacological and behavioral determinants of cocaine, methamphetamine, 3,4-methylenedioxymethamphetamine and *para*-methoxyampehtamine-induced hyperthermia" (Psychopharmacology, 2007)

The aim of this study was twofold. The first was to define the relative potencies of the most popular illicit drugs associated with hyperthermia in humans, in particular drugs which were often intentionally or unintentionally administered with MDMA. The second aim was to assess both physiological and thermoregulatory behaviour after challenge with a range of popular stimulants.

The limitation of previous studies is that they have used a small number of doses (often single), which has not allowed a pharmacologically valid comparison of the potencies of these drugs in disrupting core temperature. This is important as case studies have so far failed to reveal a clear dose-response relationship for the effect these drugs have on control of core temperature and hyperthermia. We administered 4-5 doses of each drug to rats to give a clear picture of the dose-response relationships leading to hyperthermia at a high ambient temperature.

The second limitation of previous studies is that they have not looked at the behavioural aspects of thermoregulation. This is a very important part of overall control of core

temperature, as harm minimisation depends on users behaving appropriately to cool themselves if they are hot. This paper is an extension of my honours work, which gave rats only single doses of MDMA, PMA and d-amphetamine, and only looked at behavioural thermoregulation for one hour (Jaehne et al, 2005).

1.10.2. Publication 2: "The effect of long term repeated exposure to 3,4-methylenedioxymethamphetamine on cardiovascular and thermoregulatory changes" (Psychopharmacology, 2008)

The aim of this publication was to investigate the effect of multiple doses of MDMA given on three consecutive days a week for either 6 weeks or 1 week on parameters of behavioural thermoregulation, a regimen more akin to use in humans. This work was an extension of the previous study which looked at the effect of single administration of MDMA on thermoregulatory parameters. Core temperature, heart rate and locomotor activity were also measured. In addition cortical concentrations of 5HT and DA were measured 1 week after the final dose to determine if there were any residual effects of the MDMA dosing regimens.

1.10.3. Publication 3: "Increased effects of 3,4-methylenedioxymethamphetamine (ecstasy) in a rat model of depression" (Accepted by Addiction Biology)

The aim of this publication was to investigate the effects of MDMA in Flinders Sensitive Line (FSL) rats to identify if a predisposition to depressive-like behaviour results in a modified response to the drug. This was an extension of the previous two papers into a different strain of rat, which is a model of human depression and predisposition to depression. Many long term users of ecstasy have depression, and 5HT is a common factor in depression and MDMA outcomes. We considered it important to know if depression altered thermoregulatory responses to the drug. MDMA was given at two doses and our usual model of behavioural thermoregulation was again used. In addition cortical concentrations of 5HT and DA were measured 1 week after the final dose to determine

whether any residual effects of the MDMA dosing regimens were different in FSL and SD rats. Blood and brain MDMA and MDA concentrations were also measured.

1.10.4. Manuscript 4: "A behavioural, neurochemical and proteomic analysis after treatment with 3,4-methylenedioxymethamphetamine and methamphetamine" (Prepared as manuscript for submission)

This paper indicates a change in focus, moving to investigate the long term residual effect of MDMA on the brain using a proteomic method. Proteomics has significant advantages in identifying targets of drug action. It is especially useful when the drug has multiple actions. Physiological thermoregulatory effects were also measured, as were cortical concentrations of 5HT and DA, to relate the results to the previous papers. MDMA, methamphetamine, and a combination of MDMA + methamphetamine were given twice a day for four days, and brains were removed two weeks later. Methamphetamine acted as a positive control for MDMA to demonstrate drug selective effects but is also of interest when one considers that it is the most commonly co-abused drug used with MDMA. The aim of this paper was therefore to investigate the effects of these drugs on protein expression in the cortex of rats given previously described doses which are relevant to human exposure.

2. Publication 1

Jaehne E. J., Salem A. and Irvine R. J. (2007) Pharmacological and behavioral determinants of cocaine, methamphetamine, 3,4-methylenedioxymethamphetamine and *para*-methoxyamphetamine-induced hyperthermia. Psychopharmacol (Berl) 194: 41-52

The aim of this study was to define the relative potencies of the most popular illicit drugs associated with hyperthermia in humans; methamphetamine, cocaine, MDMA and PMA, and to assess both physiological and thermoregulatory behaviour after challenge with this range of popular illicit stimulants. This work is an extension of the work done during my honours year and is the first comprehensive pharmacological study examining and comparing a range of the most popular illicit stimulants and assessing the effects on core temperature.

The current study addressed the fact that the majority of previous studies have used a limited number of doses, which has not given adequate information of the pharmacology of these compounds. This is important as case studies have so far failed to reveal a clear dose-response relationship for the effects stimulant drugs have on thermoregulation in human users. We showed clear differences in the slope of the dose-response curves of the four drugs studied for increasing T_C at a high ambient temperature. MDMA and PMA had much steeper slopes than methamphetamine and cocaine at doses higher than 12 μmol/kg, suggesting even small increases in dose could lead to much higher increases in T_C.

This paper also addresses the fact that although previous studies have investigated the effect of stimulants on the physiological control of core temperature, no other groups have used the thermal gradient model used in our papers, which is unique in that it measures both the physiological and behavioural aspects of thermoregulation simultaneously. This paper showed that rats with methamphetamine-, cocaine-, or MDMA-induced hyperthermia chose cooler areas on the runway for at least the first 30 minutes, with higher

doses leading to higher T_C in the heat and choice of lower temperatures in the gradient until T_C returned to normal. However, rats treated with PMA did not choose cool areas on the runway and T_C still returned to normal, suggesting that different aspects of thermoregulation are disrupted after administration of different stimulant drugs. There were no significant differences in cortical concentrations of 5HT, DA and their metabolites between untreated controls and any drug treatment group.

3.   Publication 2

Jaehne E. J., Salem A. and Irvine R. J. (2008) The effect of long-term repeated exposure to 3,4-methylenedioxymethamphetamine on cardiovascular and thermoregulatory changes. Psychopharmacol (Berl) 201: 161-70

The aim of this study was to investigate the effects of a long term dosing regimen of MDMA, simulating human weekend use of ecstasy, and to determine whether tolerance or sensitisation developed to both the physiological and behavioural aspects of thermoregulation. This paper represents a logical progression from Paper 1, which demonstrated the importance of behavioural aspects of thermoregulation in thermal gradient experiments, but only looked at the acute effect of a single administration of MDMA.

We have now investigated the effect of multiple administrations of MDMA given on three consecutive days a week, resembling weekend dosing in humans, for either 6 weeks or 1 week on T_C, HR, LMA and cortical concentrations of 5HT and DA. MDMA was given at either a high or normal ambient temperature. Previous studies have shown tolerance, sensitisation or no change in the effect of MDMA on different physiological parameters after repeated dosing. We showed that the dosing regimen used led to tolerance to the MDMA-induced increase in HR at both ambient temperatures, both over each of the three days in each week, as well as over the 6 week treatment period. Rats showed neither tolerance nor sensitisation to the effects of MDMA on T_C and LMA.

Giving rats' access to the thermal gradient showed differences not reported previously between rats treated for 1 week compared to 6. T_C and T_P were similar initially, when comparing between rats treated at the same ambient temperature. However, during the final hour in the thermal gradient, rats which had been treated with MDMA for 6 weeks started to show a secondary increase in T_C, with a trend towards choosing higher temperatures in

the thermal gradient. Rats which had been treated with MDMA for 6 weeks also showed

significant decreases in DOPAC and 5HIAA in the cortex, but not DA or 5HT.

4. Publication 3

Jaehne E. J., Majumder I., Salem A. and Irvine R. J. (2009) Increased effects of 3,4-methylenedioxymethamphetamine (ecstasy) in a rat model of depression. Addiction Biology (accepted 13/10/2009)

The aim of this study was to investigate the effects of MDMA in a rat model of depression to identify whether a predisposition to depressive-like behaviour results in a modified response to the behavioural, cardiovascular and neurochemical effects of the drug.

The current study compares the effects of MDMA on "non-depressed" SD rats with a genetic rat model of depression, the Flinders Sensitive Line (FSL). Although MDMA use is associated with depression in humans, no preclinical studies have attempted to clarify causality, mechanisms or any dangers associated with this link. We used a similar experimental protocol to that used in Paper 1, with only 2 doses of MDMA given per group.

Our study indicated that the effects of MDMA may well be exaggerated in the presence of pre-existing depression, which may have significant implications for health effects of this widely used drug in human populations. Hyperthermia was greater in FSL rats given MDMA at a high ambient temperature, and led to significantly more fatalities due to hyperthermia. HR was also higher throughout the whole experiment after FSL rats had been given the higher of the two MDMA doses. We also found significant decreases in cortical concentrations of 5HT, DA and their metabolites which were not shown in SD rats in any of the papers presented in this thesis.

TITLE: Increased effects of 3,4-methylenedioxymethamphetamine (ecstasy) in a rat model of depression

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ABSTRACT:

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is associated with increases in core body temperature (T_C) and depressive mood states in users. Flinders Sensitive Line (FSL) rats represent a rat model of depression, originally bred from Sprague-Dawley (SD) rats. They are more sensitive to both muscarinic and serotonergic agonists and have altered thermoregulatory responses to various drugs. To examine the link between MDMA and depression, 8 FSL and 8 SD rats were administered saline and 5 and 7.5 mg/kg MDMA. Immediately following administration, rats were confined to an area with an ambient temperature (T_A) of 30±1°C for 30 minutes, before being allowed access to a thermal gradient for four hours. Brains were removed one week after final dose of MDMA and concentrations of serotonin and dopamine measured. Treatment with MDMA at both doses led to a higher T_C in FSL rats than SD rats at high T_A ($P < 0.01$). Fatalities due to hyperthermia occurred in FSL rats after both doses, whereas all but one of the SD rats recovered well. Heart rate was also much higher after MDMA in FSL rats throughout the experiments. FSL rats showed significant decreases in all transmitters measured ($P < 0.05$). These differences between strains were not accounted for by altered blood or brain concentrations of MDMA. The results indicate that FSL rats may be more susceptible to developing MDMA-induced hyperthermia and possible damage to the brain. These findings may be of importance to human users of MDMA who also have depression.

INTRODUCTION

3,4-Methylenedioxymethamphetamine (MDMA, 'ecstasy') is an amphetamine derivative widely used in rave party and club scenes. Its use is widespread throughout the world and generally has increased over the last decade (United Nations, 2003; 2008). There are several major acute adverse effects associated with the use of MDMA, and although the incidences of these are low, the events are unpredictable and can lead to death or morbidity (Gowing et al, 2002; Williamson et al, 1997). One major adverse effect of MDMA ingestion is hyperthermia, leading to multiple morbidities and even death (Lyles and Cadet, 2003; Screaton et al, 1992). High ambient temperatures are thought to be a major contributor to these cases of hyperthermia. MDMA use has also been associated with the development of depression and memory deficits (Green et al, 2003; Parrott, 2002; Parrott et al, 2006). The mechanisms underlying both acute and longer term effects of MDMA are not fully understood but are thought to be a result of disruption of normal neurotransmitter functions. A number of neurotransmitters have been implicated but serotonin and dopamine systems seem to be the major contributors (Malberg et al, 1996; Mechan et al, 2002).

MDMA, especially at high ambient temperatures, leads to long term depletion of serotonin in the brains of rats (Fischer et al, 1995; Malberg and Seiden, 1998; Wang et al, 2004), and possibly humans (McCann et al, 1998; Volkow et al, 2001), and it has therefore been suggested that it could lead to long term depression and mood disturbances in users (Green et al, 2003). Although reports from users suggest that ecstasy use can lead to a midweek mood disturbance in the days after ecstasy use, it is not clear whether it causes depression as a long-term effect. In fact, several studies show evidence that depression may actually precede MDMA use in some users (Guillot and Greenway, 2006; Lieb et al, 2002; Soar et al, 2001). This is important, as these people could therefore have the potential to respond

differently to drugs such as MDMA which have a significant serotonergic component to their mechanism of action.

Flinders Sensitive Line (FSL) rats represent a rat model of depression. They were first bred from Sprague-Dawley (SD) rats to be more sensitive to anticholinesterase inhibitors (Overstreet et al, 1979). Importantly for this study, they were shown to have an increased hypothermic response to different drugs (Overstreet and Russell, 1982). They have also been shown to be more sensitive to both thermoregulatory and behavioural effects of serotonergic drugs, in particular those acting on $5HT_1$ receptors (Shayit et al, 2003; Wallis et al, 1988). FSL rats have also been shown to be more sensitive to the hypothermic effects of dopamine, although are less sensitive to the stereotypy effects compared to control rats (Crocker and Overstreet, 1991). Thus there are altered thermoregulatory responses to drugs in this strain, in combination with changes in neurotransmitters implicated in the effects of MDMA (Green et al, 2004a).

FSL rats have many similarities with depressed humans. Both show increased cholinergic sensitivity (Janowsky et al, 1994; Overstreet, 2002; Overstreet and Russell, 1982), altered serotonergic activity (Overstreet et al, 2005) and reduced dopamine transporter levels in the brain (Crocker and Overstreet, 1991; Meyer et al, 2001). FSL rats show extended periods of immobility in the forced swim test and this behaviour, akin to lack of motivation in depressed humans, can be reversed by clinically effective anti-depressants designed to increase brain concentrations of 5HT and noradrenaline including tricyclics, selective serotonin reuptake inhibitors (SSRI) and others (Overstreet et al, 2005). If there is a link in humans between MDMA use and pre-existing or developing depression, this animal model appears well suited to examine this link.

The aim of the current study is therefore to investigate the effects of MDMA in FSL rats to identify if a predisposition to depressive-like behaviour results in a modified response to the drug compared to SD controls. The Flinders Resistant Line (FRL) was the original

control developed alongside the FSL strain. Both Flinders lines were bred from SD rats originally and there are no differences between randomly bred SD rats and FRL rats in the parameters we are interested in (Hasegawa et al, 2006; Hildreth et al, 2008; Overstreet et al, 1990; Overstreet and Russell, 1982; Zangen et al, 1997). SD rats also provide a broader control for the effects of MDMA, as most research done looking at the effects of MDMA have used SD rats. We hypothesise that FSL rats will be more sensitive to the thermoregulatory effects of MDMA, and show differences in behavioural, cardiovascular and longer term neurochemical effects of the drug.

MATERIALS AND METHODS

Animals

8 Male Sprague-Dawley (SD) rats and 8 male Flinders Sensitive Line (FSL) rats, 7 weeks of age at the time of telemetry implant surgery, were used for testing. The rats were housed in groups of 2-3 during the experimental period, including post-operatively, with food and water available *ad libitum*. Home cage temperature was in the range 22 ± 1.5°C. All behavioural testing was conducted between 1000 and 1500 hours, when the core body temperature of rats varies little under normal conditions (Gordon, 1990). FSL rats were sourced from a colony at the University of Technology, Sydney, which is now being maintained at the University of Adelaide. All experimentation was approved by the University of Adelaide Animal Ethics Committee and followed the Australian code of practice for the care and use of animals for scientific purposes.

Equipment

The apparatus used (supplementary figure) was the same as we have used previously (Jaehne *et al*, 2005; 2007; 2008) and based on previous studies (Florez-Duquet et al, 2001; Gordon, 1987). It consists of an insulated aluminium floor (120 cm) with an actual runway

length of 72 cm divided into five zones with dimensions 14.5 by 30 cm. The runway is split into two 15 cm wide sides so that two rats can be observed simultaneously. The ends and centre divide are aluminium to eliminate signal interference between transmitters in rats (28 cm high), and there are transparent plexiglass sidewalls for observations. There is a mesh lid on top to prevent rats from escaping while keeping the T_A constant compared to previous studies in which we did not use the lid. Rats are initially confined to the warmest zone by a removable barrier, so that the confinement area has dimensions 14.5 by 15 cm, with a lid 14 cm above the floor. At one end of the floor during experiments is a metal container filled with ice, and at the other end underneath the floor is a heat box set at 62°C. Thermocouple wires are attached between the underside of the floor and a layer of Styrofoam insulation, at the centre of each zone. The equipment was allowed to equilibrate to the required floor temperatures for at least one hour prior to each experiment. The floor temperature for the five zones were 11 ± 1, 17.5 ±1, 22.5 ± 1, 29 ± 1.5 and 39.5 ± 1.5°C, and were measured continuously throughout each experiment.

Preparation and Administration of Drugs

(±)-MDMA was given as the hydrochloride salt and was dissolved in 0.9% saline to give concentrations of 5 or 7.5 mg/ml. The drug was administered at 1 ml/kg via i.p. injection at doses of 5 or 7.5 mg/kg. These doses were chosen based on our previous work (Jaehne et al, 2005; 2007) and a within subjects design was used. All rats first received a control treatment, consisting of the same dose volume of saline only, followed by MDMA at 5 mg/kg one week later and 7.5 mg/kg a further week later. Doses were given with one week between administration to allow sufficient time for the drug to be cleared from their system (Law and Moody, 1994). The lower dose was given first as this dose has been shown to not be neurotoxic in SD rats (Cappon et al, 1998; Green et al, 2003; Kita et al, 2003) and a previous similar dosing regimen showed no neurotoxic effect in SD rats (Jaehne et al,

2007). MDMA was obtained from The Australian Government Analytical Laboratories (Sydney, Australia).

Data Acquisition

Rats were surgically implanted with telemetry devices (TA11CTA-F40, Data Sciences International), which measure core body temperature (T_C), locomotor activity (LMA) and heart rate (HR), as reported previously (Bexis et al, 2004). The implants were placed into the rats abdominal cavity under anaesthesia (sodium pentobarbital, 60 mg/kg). Two weeks recovery from surgery was allowed before rats underwent any injection treatments. Radio receivers, placed to the side of the runway, received information from the implants and transferred it to a computer which recorded the data using Dataquest LabPro software (Data Sciences International). Data was recorded every two minutes over the experimental period.

Experimental Protocol

During the week before testing began, and starting one week after surgery, rats were habituated to the apparatus by being placed in the warm area with an ambient temperature of $30 \pm 1°C$ for 30 minutes on two separate occasions. They were also placed in the thermal gradient on two separate occasions, once directly from the home cage and once after being in the warm ambient temperature. On experimental days, rats were taken from their home cage, administered either saline or drug, and placed in to the warm ambient temperature for 30 minutes as we have done previously (Jaehne et al, 2005; 2007; 2008). At the end of the 30 minutes (time (t) = 0), rats were allowed access to the thermal gradient for four hours to choose their preferred floor temperature. Temperature preference (T_P) was recorded as the zone each rat was in at the end of every two-minute period. T_C, LMA and HR were recorded via telemetry every two minutes.

Neurotransmitter Analysis

One week after the final dose of MDMA, animals were anaesthetised (sodium pentobarbital 60mg/kg), decapitated, and their brain removed and frozen. The cerebral cortex was dissected from one half of the frozen brain samples of each rat and prepared for HPLC as described by Callaghan et al (2006) and concentrations of 5-HT, 5-HIAA, DA and dihydroxyphenyl acetic acid (DOPAC) measured. This data was compared to animals of the same age and strains which had undergone no previous treatments. The HPLC- EC system consisted of a Shimadzu (Kyoto, Japan) HPLC system controller (CBM-20A) and Antec Leyden Decade II electrochemical detector housed at 30°C, potential of 0.7 V and a range of 10 nA. The mobile phase consisted of: NaH_2PO_4 103 mM, octanesulphonic acid 0.5 mM, EDTA 0.1 mM and 10% methanol, pH 3.8. The mobile phase was delivered at a flow rate of 0.4 ml/min. Compounds of interest were separated using a 250 x 4.6 mm 5μ C18 column (Beckman) and sampling was recorded using a Shimadzu LCsolution workstation.

Pharmacokinetic Analysis

A separate group of 18 SD and 18 FSL rats were used to determine the concentration-time profile of MDMA and the metabolite 3,4-methylenedioxyamphetamine (MDA) in blood and brain. Rats were dosed with MDMA (7.5 mg/kg) and placed back in to their home cage. Rats were anesthetized and blood taken by cardiac puncture at time points 30, 60, 120 and 240 min (n = 4-5/strain). Brains were also removed immediately following blood sampling and stored at -70ºC. MDMA and MDA concentrations in brain samples were determined using the method described above for determining neurotransmitter concentrations. MDMA and MDA concentrations in blood sample were determined using a previously published method (Michel et al, 1993), with the HPLC system used the same as that for brain analyses, but with a potential of 1.2 V and a range of 20 nA, detector housed

at 25ºC. The mobile phase consisted of: sodium acetate 0.1M and 12.5% methanol, pH 4.25. The mobile phase was delivered at a flow rate of 0.12 ml/min. Compounds of interest were separated using a 100 x 2.1 mm 3µ HPC18 column (Alltima).

Statistical Analysis

All calculations and analysis were done using Graph Pad Prism software. Mean values for T_C, T_P, HR and LMA over time were calculated for the time periods –30-0, 0-30, 30-60, 60-120, 120-180 and 180-240 minutes and analysed between all treatments using two-way ANOVA's with Bonferoni's post hoc tests between treatments. Cortical concentrations of neurotransmitters were compared to untreated age matched controls of the same strain, which had not undergone any of the experimental procedures, using unpaired, 2-tailed t-tests. Blood and brain concentrations of MDMA and MDA were compared between strains by performing unpaired, 2-tailed t-tests on the AUC of the concentration-time curves as well as apparent $t_{1/2}$ and Cmax. All results are presented as mean ± SEM. $P < 0.05$ was taken as significant for all analyses. Data recorded in the 25-30 minutes prior to death was included for rats which died.

RESULTS

Effects of MDMA treatment in SD and FSL rats at high ambient temperature

Fatalities A pilot group of 4 FSL rats were treated with 10 mg/kg MDMA, as this dose had been shown to be safe using our experimental protocol in SD rats (Jaehne *et al*, 2005). However, 3 out of these 4 rats died due to severe hyperthermia during confinement to high ambient temperature. Therefore, due to ethical concerns, the doses were reduced. At 5 mg/kg 2 out of 8 FSL rats died and at 7.5 mg/kg 3 out of the remaining 6 MDMA treated FSL rats died. In contrast, the only fatality in the SD strain was 1 out of 8 after treatment with 7.5 mg/kg MDMA. The maximum T_C achieved prior to death was 43.55 ± 0.13 ºC.

These animals, in every case, lost mobility and died in the last 5 minutes of the 30 minute exposure to high ambient temperature.

Core Temperature Table 1 show T_C data from rats while they were confined to the high T_A. Baseline temperature of FSL rats was significantly higher than SD rats before all treatments ($P < 0.0001$). One-way ANOVA of T_C and change in T_C both showed significant effects of treatment ($P < 0.0001$). Both doses of MDMA led to highly significant increases compared to saline in both SD and FSL rats ($P < 0.001$). Maximum T_C achieved was significantly higher in FSL rats compared to SD rats after both 5 mg/kg ($P < 0.001$) and 7.5 mg/kg MDMA ($P < 0.01$), while change in T_C was significantly greater in FSL rats after 5 mg/kg MDMA ($P < 0.05$).

Acute effects of MDMA treatment in SD and FSL rats during behavioural thermoregulation

Core Temperature Figure 1 shows the effects of treatment on T_C. Two-way ANOVA of mean T_C showed a significant interaction effect ($P < 0.0001$, $F = 5.196$) and significant effects of treatment ($P < 0.0001$, $F = 23.77$) and time ($P < 0.0001$, $F = 159.8$). In SD rats, T_C was significantly higher after 7.5 mg/kg MDMA than saline during confinement ($P < 0.01$). It was also significantly lower than after saline treatment during the time period 30-60 minutes after 5 mg/kg ($P < 0.001$) and 7.5 mg/kg MDMA ($P < 0.05$).

In FSL rats, T_C was significantly higher than saline treatment after 5 mg/kg MDMA during confinement ($P < 0.05$), and lower during the time periods 30-60 minutes ($P < 0.001$) and 60-120 minutes ($P < 0.001$). 7.5 mg/kg MDMA led to a significant increase compared to saline treatment during confinement ($P < 0.01$), and decreases during time periods 30-60 minutes ($P < 0.001$), 60-120 minutes ($P < 0.001$) and 120-180 minutes ($P < 0.01$). 7.5 mg

MDMA also led to a significantly higher mean T_C compared to 5 mg/kg MDMA during the time period 0-30 minutes ($P < 0.05$).

FSL rats also showed significant differences to SD rats when treated at the same dose of MDMA. FSL rats had a higher mean T_C after saline treatment during the time periods 30-60 minutes ($P < 0.05$), 60-120 minutes ($P < 0.001$) and 120-180 minutes ($P < 0.001$). FSL rats also had a higher mean T_C after 5 mg/kg MDMA during confinement ($P < 0.01$) and after both doses of MDMA during the time period 0-30 minutes (5 mg/kg $P < 0.001$; 7.5 mg/kg $P < 0.05$).

Temperature Preference Figure 2 shows the effect of treatment on T_P. Two-way ANOVA of mean T_P showed significant interaction effect ($P = 0.0006$, $F = 2.548$) and significant effects of treatment ($P < 0.0001$, $F = 11.09$) and time ($P < 0.0001$, $F = 13.76$). In SD rats, T_P was significantly lower than saline treatment after both doses of MDMA during the time period 0-30 minutes (5 mg/kg $P < 0.05$; 7.5 mg/kg $P < 0.01$).

FSL rats treated with 5 mg/kg MDMA had lower T_P than after saline treatment during the time period 120-180 minutes ($P < 0.05$). Treatment with 7.5 mg/kg MDMA in FSL rats led to lower T_P than saline during the time periods 0-30 minutes ($P < 0.05$), 120-180 minutes ($P < 0.001$) and 180-240 minutes ($P < 0.001$), lower T_P than 5 mg/kg MDMA during 180-240 minutes ($P < 0.05$), and also lower T_P than SD rats during 120-180 minutes ($P < 0.01$) and 180-240 minutes ($P < 0.05$).

Heart Rate Figure 3 shows the effect of treatment on HR. Two-way ANOVA of mean HR showed significant effects of treatment ($P < 0.0001$, $F = 60.05$) and time ($P < 0.0001$, $F = 22.28$). In SD rats, HR was significantly higher than saline treatment after 7.5 mg/kg MDMA during confinement ($P < 0.01$), 30-60 minutes ($P < 0.05$), 60-120 minutes ($P <$

0.05) and 120-180 minutes (P < 0.05), and after 5 mg/kg during the time period 60-120 minutes (P < 0.05).

FSL rats treated with 5 mg/kg MDMA showed significantly higher HR than saline during confinement (P < 0.001), 0-30 minutes (P < 0.001), 30-60 minutes (P < 0.01), 120-180 minutes (P < 0.01) and 180-240 minutes (P < 0.05), as well as significantly higher HR than SD rats when given the same dose during confinement (P < 0.05), 0-30 minutes (P < 0.001), 120-180 minutes (P < 0.01) and 180-240 minutes (P < 0.05). FSL rats given 7.5 mg/kg had a significantly higher HR than saline at all time periods (confinement P < 0.01; all other time periods P < 0.001). HR was also significantly higher than after 5 mg/kg during the time period 180-240 minutes (P < 0.05) and compared to SD rats given the same dose during the time periods 0-30 minutes (P < 0.001), 30-60 minutes (P < 0.05), 60-120 minutes (P < 0.01), 120-180 minutes (P < 0.001) and 180-240 minutes (P < 0.001).

Locomotor Activity Figure 4 shows the effect of treatment on LMA. Two-way ANOVA of mean LMA showed a significant interaction effect (P < 0.0001, F = 6.257) and significant effects of treatment (P < 0.0001, F = 19.79) and time (P < 0.0001, F = 34.91). In SD rats LMA was significantly higher than saline after 5 mg/kg MDMA during confinement (P < 0.001) and 0-30 minutes (P < 0.05) and after 7.5 mg/kg MDMA during confinement (P < 0.001), 0-30 minutes (P < 0.001), 30-60 minutes (P < 0.001) and 60-120 minutes (P < 0.05). LMA was also higher after 7.5 mg/kg MDMA than 5 mg/kg MDMA in SD rats during the time periods 0-30 minutes (P < 0.05) and 30-60 minutes (P < 0.05).

In FSL rats, LMA was significantly higher than saline during confinement after both 5 mg/kg (P < 0.001) and 7.5 mg/kg MDMA (P < 0.001). Higher LMA was seen in FSL compared to SD rats during confinement after 5 mg/kg MDMA (P < 0.001) and 7.5 mg/kg MDMA (P < 0.001).

Long term effects of MDMA treatment in SD and FSL rats

Cortical Concentrations of Neurotransmitters Table 2 shows the concentrations of 5HT, 5HIAA, DA and DOPAC in rats treated with MDMA, and age matched controls of the same strain, in the cortex of rats 1 week after the final administration of MDMA. There were no significant changes in cortical concentration of any of the neurotransmitter measured in SD rats compared to controls using unpaired, 2 tailed t-tests. In contrast, FSL rats showed significant decreases compared to untreated controls of all neurotransmitters measured (5HT P = 0.024; 5HIAA P = 0.046; DA P = 0.0014; DOPAC P = 0.011).

MDMA and MDA concentrations in SD and FSL rats

Blood and brain concentrations of MDMA and its primary active metabolite MDA in both rat strains were identical (Fig 5). MDMA AUCs for SD rats were 6209 ± 2006 in blood and 1062000 ± 380732 in brain, and were not significantly different from FSL rats 5622 ± 1327 in blood and 845657 ± 212228 in brain (blood P = 0.816; brain P = 0.638). Apparent $t_{1/2}$ of MDMA in SD rats was 48.53 ± 1.04 min in blood and 28.03 ± 7.99 min in brain and in FSL rats was 43.58 ± 8.74 min in blood and 39.76 ± 1.99 min in brain (blood P = 0.723; brain P = 0.204). Cmax of MDMA in SD rats was 3239 ± 933 ng/ml in blood and 10040 ± 3198 ng/g in brain and in FSL rats was 2226 ± 498 ng/ml in blood and 7653 ± 1363 ng/g in brain (blood P = 0.376; brain P = 0.512). Likewise the metabolic ratios were not significantly different between strains. MDA AUC's for SD's were 1020 ± 364 in blood and 594439 ± 210731 in brain, compared to 1086 ± 265 in FSL blood and 455195 ± 108045 in FSL brain (blood P = 0.888; brain P = 0.578). These data indicate that the pharmacodynamic differences observed between the strains could not be explained by strain differences in pharmacokinetics, metabolism or access to the brain.

DISCUSSION

This study is the first to demonstrate enhanced effects of MDMA in an animal model of depression when compared to control animals. When MDMA was administered at a high T_A, core temperature rose to a much higher level in FSL rats compared to SD's, which also led to a higher rate of fatalities due to hyperthermia. This may be explained by T_C at the start of confinement to high T_A, which was nearly 1ºC higher in this strain. However, the change in T_C after both doses of drug was also greater in FSL rats. Home cage temperatures prior to experimentation were identical for both strains. This finding is consistent with previous data reporting baseline T_C during the day in FSL rats (Shiromani et al, 1991; Wallis et al, 1988). The most likely reason for the increase in T_C in FSL rats before measurements have started is due to the stress of being handled. Unrecorded observations from this study are that FSL rats did not habituate to handling as well as SD rats. It has been shown that acute handling stress can lead to different hormonal responses in FSL rats (Zambello et al, 2008). It has also been shown in several different strains of rats that stress due to restraint or other experimental procedures can lead to an acute increase in T_C (Gordon, 1990) and HR (Irvine et al, 1997; McDougall et al, 2005).

To understand the reasons for the greater T_C increase in FSL rats the current proposed mechanism of action of MDMA on thermoregulation needs to be considered. It has been shown that activation of postsynaptic 5-HT$_{2A}$ receptors using (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) causes hyperthermia through cutaneous vasoconstriction in both rats and rabbits (Blessing and Seaman, 2003), while inhibitory presynaptic 5-HT$_{1A}$ receptor activation with 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) leads to vasodilation and a fall in body temperature in rabbits (Ootsuka and Blessing, 2003). It has therefore been suggested that MDMA, after releasing 5-HT in the

CNS, increases cutaneous vasoconstriction by these mechanisms leading to hyperthermia at normal to high T_A (Blessing et al, 2003).

FSL rats have been shown to be more sensitive to the hypothermic effects of cholinergic (Overstreet et al, 1998), dopaminergic (Crocker and Overstreet, 1991), and serotonergic (Wallis et al, 1988) drugs compared to FRL rats. In particular, FSL rats show increased hypothermic effects after both the $5HT_2$ receptor antagonist cryoheptadine, and the $5HT_1$ agonist l(m-chlorophenyl) piperazine dihydrochloride (mCPP) compared to FRL rats (Wallis et al, 1988). These results, and others showing the effect on behaviour compared to SD controls (Shayit et al, 2003), suggest that FSL rats are more sensitive to serotonergic agonists compared to both FRL and SD rats. Like other drugs acting on the serotonergic system, MDMA also leads to hypothermia at low T_A (Jaehne et al, 2005). If MDMA was administered under these conditions, one would expect the hypothermic response to be greater in FSL than SD rats. Therefore the increased response to the hyperthermic effects of MDMA as we saw here is consistent with our current understanding of the mechanisms of action of this drug in rats.

When rats were allowed access to the thermal gradient to behaviourally thermoregulate, both strains showed a trend towards choosing cooler areas on the runway with increasing dose and T_C as we have seen in these type of experiments previously (Jaehne et al, 2007). The T_C of both strains of rats showed a similar pattern here after MDMA treatment, falling below baseline as soon as they were allowed in the runway, then returning to normal by the end of the four hours. This was again consistent with previous results in this laboratory (Jaehne et al, 2005; 2007; 2008). However, when FSL rats were treated with 7.5 mg/kg MDMA, they showed a slower recovery in T_C, despite choosing the cooler areas of the runway, suggesting a possible continued disruption to autonomic thermoregulation. Preferred temperature on the runway continued to show no differences between strains or doses until the final two hours in the runway, when FSL rats treated with the high dose of

MDMA chose the cooler areas on the runway again, which may have been what kept their T_C the same as other groups during this period. In humans, the pattern of use of MDMA and associated activities, such as dancing, generally extend for a number of hours. Thus, delayed effects of MDMA on thermoregulation may be important in understanding adverse effects in humans.

The HR of SD rats showed a trend towards increasing with increasing doses for most of the experiment which is consistent with previous results (Jaehne et al, 2005; 2008). While baseline HR was the same in both strains, HR increased to a higher level in FSL rats after all doses compared to SD rats. FSL rats also showed significant increases in HR compared to saline after both doses of MDMA for the whole time data was collected. This is consistent with a previous study which showed that there were no differences in the diurnal cycle of HR and mean arterial pressure (MAP) between FSL and SD rats, but that when rats were given the $5HT_1$ agonist 8-OH-DPAT, HR and MAP of FSL rats rose to a greater level than SD rats (Hildreth et al, 2008).

Cardiovascular physiology is important in mammals when they must adapt to changes in T_A's. When core temperature is being maintained at a set point, cardiovascular responses to cold T_A's are closely related to the mechanism for heat production in rats (Chambers et al, 2000), which increases in a parallel fashion with HR. It has been shown in non-human primates that disruption of the preoptic anterior hypothalamus by direct cooling leads to high HR and T_C, while warming leads to low HR and T_C (Morishima and Gale, 1972). HR may therefore provide an indirect indication of heat production and disrupted autonomic thermoregulation. This could suggest that the higher HR seen in FSL rats throughout the time in the thermal gradient indicates that abnormal heat production is occurring. This could then explain why these rats return to cooler areas on the runway during the final 2 hours without any major changes in T_C observed.

During confinement to the high T_A both strains showed very low LMA after saline and very high LMA after both doses of MDMA, consistent with our previous studies (Jaehne et al, 2005; 2007; 2008). Higher LMA continued to be seen after the high dose of MDMA throughout the time in the thermal gradient in both strains. LMA was also much higher in FSL rats compared to SD rats during confinement to the high T_A, which may have also contributed to the higher T_C and HR seen in these animals. It is unclear why LMA should be so much higher in these animals. FSL rats have been previously shown to display significantly lower LMA in a photocell cage compared to SD rats (Matthews et al, 1996) and previous papers have shown varying behavioural responses to different drugs. Cryoheptadine was shown to increase behavioural measurement slightly while mCPP decreased behaviour (Wallis et al, 1988) compared to FRL rats and Shayit et al (2003) showed intensified activity, hind-leg abduction and pivoting induced by 8-OH-DPAT compared with SD rats. There has been limited studies investigating the effects of other monoamines, however, Crocker et al (1991) have shown decreased stereotypy behaviour in FSL rats compared to FRL rats after administration of a DA agonist. The increased activity in the current experiments could be interpreted partly as escape behaviour from a hot, stressful area (Gordon, 1990), on top of the stimulatory effects of MDMA.

Our results indicate that differences in the pharmacokinetics of MDMA between the two strains are not responsible for the differences in thermoregulation and cardiac effects seen between SD and FSL rats. When we looked at the concentration of MDMA and MDA at different time points there were no differences in the curves seen in either blood or brain concentrations. Both whole blood and cortex concentrations measured in this study are comparable to levels reported by other workers in the same tissue in rats given a similar dose (Baumann et al, 2009; Hamida et al, 2009; Upreti and Eddington, 2008). Any differences seen could possibly be a result of the different extraction and detection

techniques used, as we used HPLC-EC compared to the more sensitive GC-MS used in the other studies, or due to use of different strains of rat. We decided to measure MDA concentration in this study, as well as MDMA, because it is the main metabolite of MDMA in rats and it has also been implicated in serotonergic neurotoxicity (Monks et al, 2004). As no differences were shown in these parameters, it now appears appropriate that future studies should be conducted to investigate any differences in brain mechanisms involved in the effects of MDMA. Comparisons between SD and FSL rats with respect to MDMA and neurotransmitter concentrations in different brains regions, neurotransmitter kinetics and specific receptors underlying the effects of MDMA would now be important.

Baseline tissue concentrations of 5HT, 5HIAA, DA and DOPAC in the cortex were similar in untreated SD rats compared to untreated FSL rats. This is in contrast to the only other data of this type in FSL rats, which showed that FSL rats had significantly higher tissue concentration of 5HT and 5HIAA in the prefrontal cortex (Zangen et al, 1997). The same group did however show that there were no differences in DA and DOPAC in the prefrontal cortex of FSL rats compared with SD rats (Zangen et al, 1999). Differences in methods used may contribute to differences in results. Zangen et al (1997; 1999) used tissue punches, compared to our use of whole cortex sample. Several contrasting studies have actually shown no difference in extracellular 5HT or 5HIAA in the nucleus accumbens of FSL rats (Dremencov et al, 2005; Zangen et al, 2001) and decreased extracellular concentrations of DA and DOPAC in this region compared to SD rats (Dremencov et al, 2005; Yadid et al, 2001; Zangen et al, 2001).

After the treatment and dosing regimen used in this study, cortical tissue concentration of neurotransmitters measured did not change in SD rats. This is consistent with previous work in our laboratory using similar dosing regimens (Jaehne et al, 2007; 2008). In contrast, FSL rats treated with MDMA showed decreases in all neurotransmitters

measured. This could suggest that FSL rats are more susceptible to the neurotoxic effects of MDMA. FSL rats may be more susceptible to damage caused by oxidative stress after MDMA administration. It is clear that oxidative stress plays a role in the long term effects of MDMA (Alves et al, 2009), a role of oxidative stress in the shorter term effects of MDMA is unclear at present, and has not been investigated in FLS rat. However, as only 3 FSL rats survived all the treatments in this study to be used in this analysis, it is difficult to be confident about this observation. More rats could not be used due to ethical concerns about the lethality rate of the treatment used. The result could also reflect the decreases seen after chronic anti-depressant treatment seen in other papers (Zangen et al, 1997; 1999). However, these decreases were normalisations of previously elevated concentrations, and likely represent a different drug effect.

In conclusion, we have shown that MDMA treatment at a high ambient temperature leads to a higher T_C and more fatalities due to hyperthermia in FSL compared to SD rats. We also showed greater MDMA-induced increases in LMA and HR at the high T_A, as well as continued elevated HR and differences in behavioural thermoregulation in a thermal gradient in FSL rats. Decreases in neurotransmitter concentration were also confined to MDMA treated FSL rats. These results demonstrate for the first time that in a rat model of depression, these animals show a higher susceptibility to developing MDMA-induced hyperthermia and depletion of neurotransmitters in the brain. We have clearly established that these differences were not due to pharmacokinetic differences, and are likely to be due to cellular mechanisms in the brain which will be studied further in the future. Considering the close association between MDMA use and depression in humans, and the difficulty in assigning cause and effects, further investigation into the relationship between depression and MDMA-induced drug effects are warranted in this animal model.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that, except for income received from the primary employer, no financial support or compensation has been received from any individual or corporate entity for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Miss Jaehne had a major input in the experimental design, conducted all telemetric studies and part of the pharmacokinetic studies, as well as conducting all of the statistical analysis and graphical presentation of the data and prepared the manuscript for submission.

Dr Majumder was involved in the experimental design, conducted neurochemical analyses and part of the pharmacokinetic studies, and contributed to the interpretation of the data collected and preparation of the manuscript.

Dr Salem was involved in the experimental design and contributed to the interpretation of the data collected and preparation of the manuscript.

Associate Professor Irvine was involved in the experimental design and contributed to the interpretation of the data collected and preparation of the manuscript.

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TABLES

Core temperature changes during confinement to a high ambient temperature

Strain/Treatment	Temperature (ºC)		
	Baseline	Maximum	Change
SD Saline	37.60 ± 0.07	39.67 ± 0.04	2.07 ± 0.09
SD 5 mg/kg MDMA	37.66 ± 0.12	40.96 ± 0.36	3.34 ± 0.37
SD 7.5 mg/kg MDMA	37.75 ± 0.20	41.69 ± 0.18	4.08 ± 0.15
FSL Saline	38.36 ± 0.09	40.47 ± 0.08	2.13 ± 0.09
FSL 5 mg/kg MDMA	38.67 ± 0.10	42.91 ± 0.27***	4.28 ± 0.30*
FSL 7.5 mg/kg MDMA	38.48 ± 0.12	43.02 ± 0.15**	4.70 ± 0.07

Table 1. Baseline core temperature (T_C at t=-30 min), and maximal core temperature achieved and maximal change in core temperature (compared to t=-30min) during 30 minutes at high ambient temperature (30 ± 1ºC) before rats were allowed in the thermal gradient. * Indicates significant difference compared to SD rats treated with same dose, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. See text for other significant differences. Data was analysed using one-way ANOVA's with Tukey's post hoc tests, n = 3-8/group.

Cortical neurotransmitter concentrations (ng/mg tissue)

Strain	5HT	5HIAA	Dopamine	DOPAC
SD untreated	0.76 ± 0.05	0.73 ± 0.04	0.29 ± 0.07	0.040 ± 0.012
SD treated	0.84 ± 0.09	0.66 ± 0.06	0.31 ± 0.10	0.030 ± 0.010
FSL untreated	0.90 ± 0.06	0.83 ± 0.03	0.29 ± 0.04	0.035 ± 0.003
FSL treated	$0.62 \pm 0.01*$	$0.72 \pm 0.02*$	$0.085 \pm 0.008**$	$0.014 \pm 0.001*$

Table 2. Cortical concentrations of 5HT, 5HIAA, DA and DOPAC measured 1 week after final dose of MDMA (treated), and in untreated control rats of the same age and strain. All data represent mean $\pm$ SEM (n = 3-8/group). * Indicates significant difference from untreated control using 2-tailed unpaired t-tests (* $P < 0.05$; ** $P < 0.01$).

FIGURE LEDGENDS

Figure 1. Effect of confinement to high ambient temperature (30 ± 1ºC) after administration of saline and MDMA 5 and 7.5 mg/kg followed by 4 h in a thermal gradient on core body temperature in SD and FSL rats. Column graphs (bottom) represent the mean for different time periods of the corresponding line graphs (top). All data represent mean ± SEM (n = 3-8/group). Data was measured every 2 min but time points for only every 6 min are shown for the 1st 90 min, and for only every 10 min for the final 3 hours for clarity. Drug was given at t = -30 min, and animals were allowed access to the thermal gradient at t = 0. * Indicates significant difference to saline and # indicates significant difference to 5 mg/kg MDMA in the same strain. ^ Indicates significant difference to SD rats treated with the same dose. Data was analysed using a two-way ANOVA with Bonferoni's post hoc test.

Figure 2. Effect of confinement to high ambient temperature (30 ± 1ºC) after administration of saline and MDMA 5 and 7.5 mg/kg followed by 4 h in a thermal gradient on preferred temperature in SD and FSL rats. Column graphs represent the mean for different time periods. All data represent mean ± SEM (n = 3-8/group). Drug was given at t = -30 min, and animals were allowed access to the thermal gradient at t = 0. * Indicates significant difference to saline and # indicates significant difference to 5 mg/kg MDMA in the same strain. ^ Indicates significant difference to SD rats treated with the same dose. Data was analysed using a two-way ANOVA with Bonferoni's post hoc test.

Figure 3. Effect of confinement to high ambient temperature (30 ± 1ºC) after administration of saline and MDMA 5 and 7.5 mg/kg followed by 4 h in a thermal gradient on heart rate in SD and FSL rats. Column graphs (bottom) represent the mean for different time periods of the corresponding line graphs (top). All data represent mean ± SEM (n = 3-

8/group). Data was measured every 2 min but time points for only every 6 min are shown for the 1st 90 min, and for only every 10 min for the final 3 hours for clarity. Drug was given at t = -30 min, and animals were allowed access to the thermal gradient at t = 0. ***** Indicates significant difference to saline and **#** indicates significant difference to 5 mg/kg MDMA in the same strain. **^** Indicates significant difference to SD rats treated with the same dose. Data was analysed using a two-way ANOVA with Bonferoni's post hoc test.

Figure 4. Effect of confinement to high ambient temperature (30 ± 1ºC) after administration of saline and MDMA 5 and 7.5 mg/kg followed by 4 h in a thermal gradient on locomotor activity in SD and FSL rats. Column graphs (bottom) represent the mean for different time periods of the corresponding line graphs (top). All data represent mean ± SEM (n = 3-8/group). Data was measured every 2 min but time points for only every 6 min are shown for the 1st 90 min, and for only every 10 min for the final 3 hours for clarity. Drug was given at t = -30 min, and animals were allowed access to the thermal gradient at t = 0. ***** Indicates significant difference to saline and **#** indicates significant difference to 5 mg/kg MDMA in the same strain. **^** Indicates significant difference to SD rats treated with the same dose. Data was analysed using a two-way ANOVA with Bonferoni's post hoc test.

Figure 5. Blood (a) and brain (b) concentrations of MDMA and MDA over time in SD and FSL rats. Rats were administered MDMA 7.5 mg/kg and sacrificed for removal of blood and brain at each time point (n = 5 at t = 30, 60 min; n = 4 and t = 120, 240 min). Data was analysed using unpaired 2-tailed t-tests between strains.
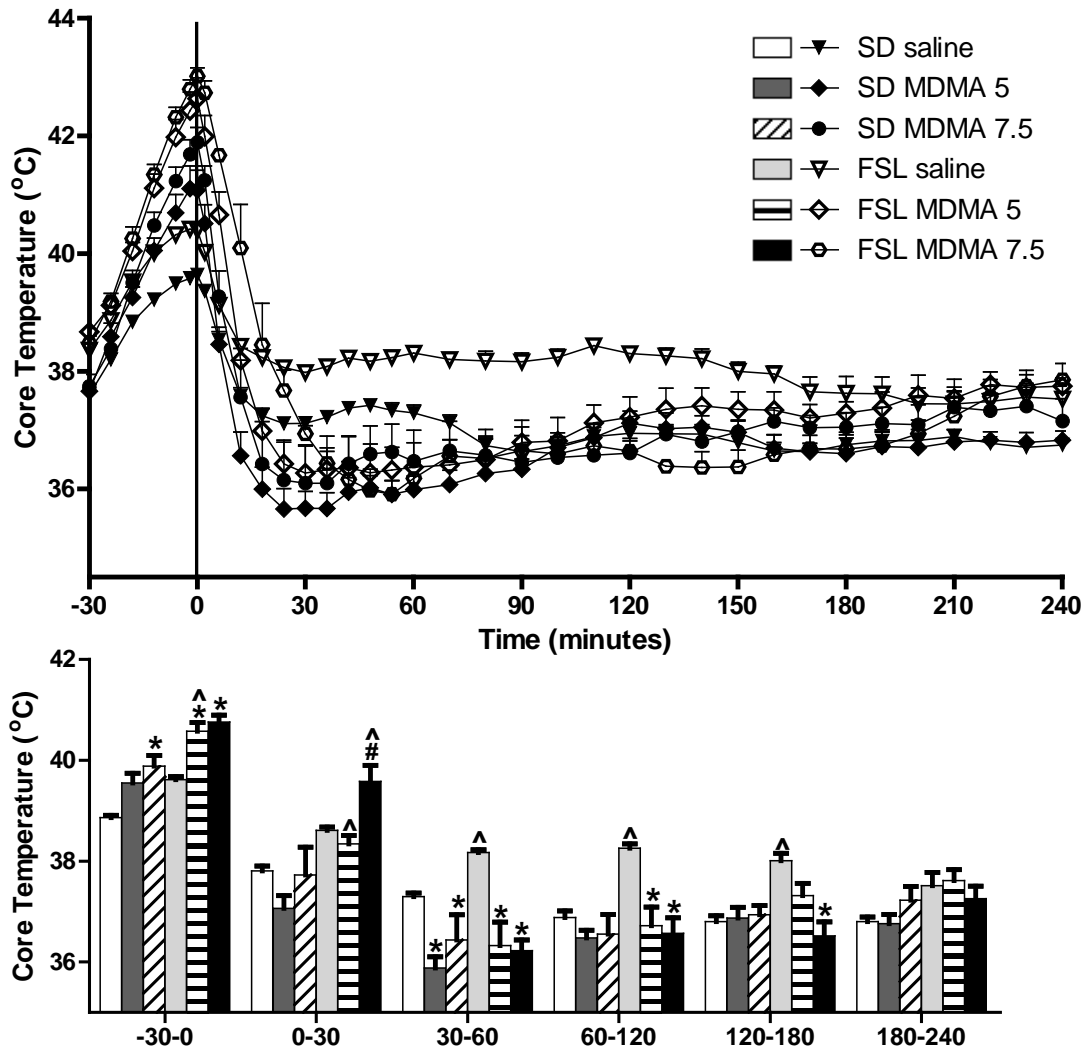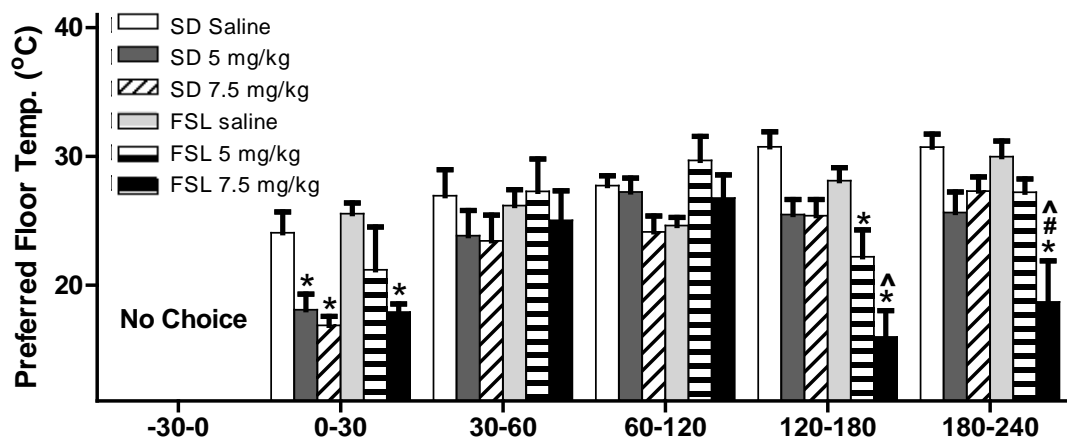
FIGURES

Figure 1

Figure 2
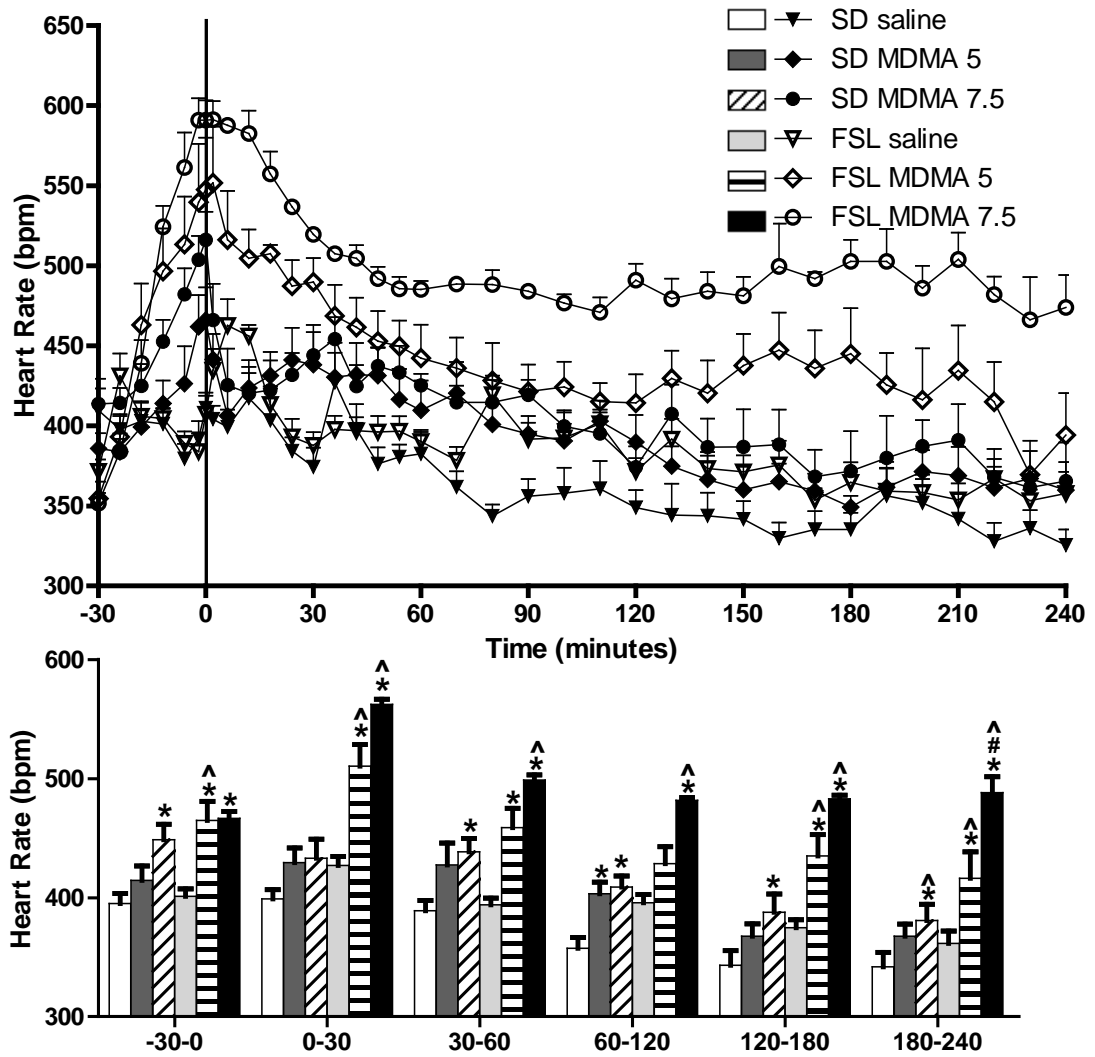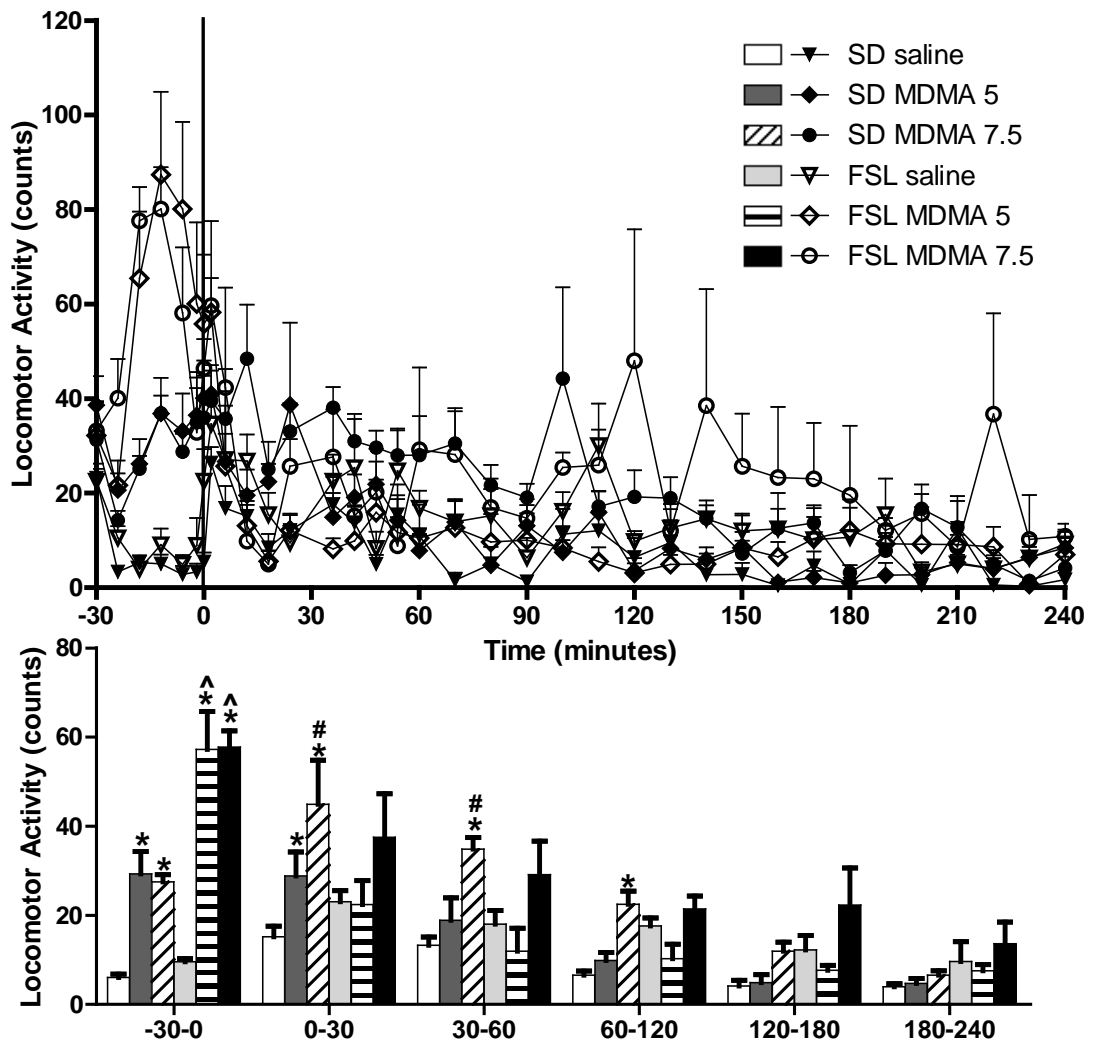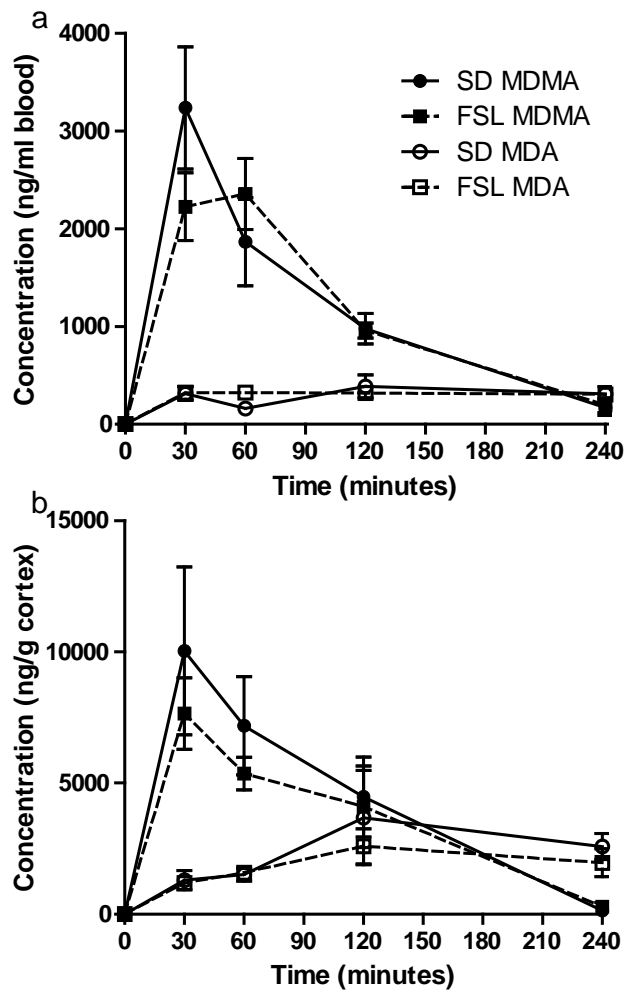
Figure 3

Figure 4

Figure 5

Supplementary Figure
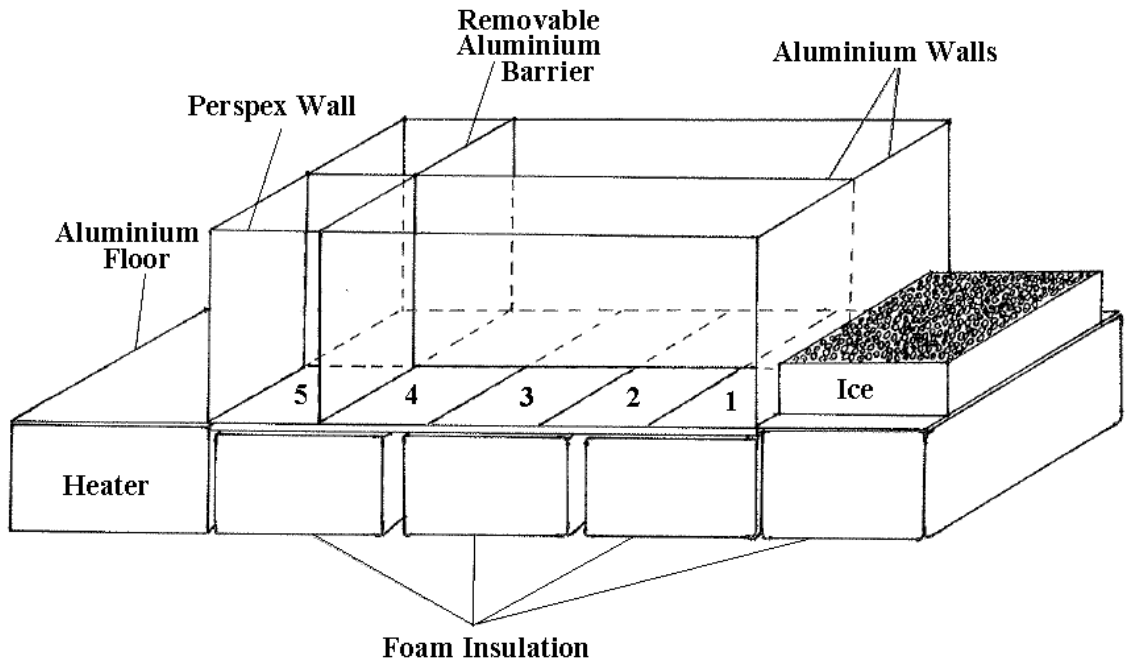
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Jaehne E. J., Colella A.D., Penno M.A.S., Hoffmann P. and Irvine R. J.

A behavioural, neurochemical and proteomic analysis after treatment with 3,4-methylenedioxymethamphetamine and methamphetamine.

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This paper shifts in focus somewhat to the first three studies presented. Only basic neurochemical analysis was conducted in the previous papers, looking at cortical concentrations of 5HT, DA and their metabolites one week after drug administration. The aim of the current study was to investigate the effects of MDMA and methamphetamine on the brain further, by conducting a pilot proteomic analysis to find any changes in protein expression caused by these drugs. The rationale was based on the lack of clear evidence of initiating mechanisms of neurotoxicity using other methods.

The aim of this pilot study was to determine whether there is a relationship between acute thermoregulatory effects, neurochemical changes and the proteomic profile in the cortex of rats administered MDMA and methamphetamine. We have therefore investigated the effects of these drugs on protein expression in the cortex of rats given previously described doses which are relevant to human exposure, using 2-dimensional electrophoresis (2-DE), in combination with powerful image analysis software and mass spectrometry (MS). Concurrent measures of thermoregulatory, behavioural and neurochemical effects of the drugs were also undertaken. Synaptosomal preparations were used as loss of terminal markers is one of the first and most robust indications of MDMA effects on the brain. Methamphetamine was used mostly as a positive control for damage to the brain, but also because MDMA and methamphetamine are often co-administered, and are both implicated in short and long term detrimental effects on the brain. To our knowledge this is the first

study to look at the effect of MDMA and MDMA + methamphetamine in combination on the proteomic profile of the rats' cortex.

When looking at the results, there did not seem to be a relationship between the proteomic profile and other established changes caused by these drugs in this study, although more comprehensive studies using a range of doses, dosing regimens, brain regions and time from last administration to removal of brain are warranted. This approach could lead to the identification of biomarkers or drug targets which would aid in early prediction or treatment of MDMA and/or methamphetamine adverse effects on brain function.

Ten proteins were identified which showed a change in abundance between at least two of the treatments. One of these spots was identified as showing a higher abundance in MDMA treated rats compared to saline treated controls, and this difference was much larger than any other comparisons between treatment groups. This spot was identified as glutathione transferase omega-1 (GSTO1) from the 2D gel. An antibody for this protein was only recently found and western blot analysis was conducted to confirm the above finding. Unfortunately, there was no difference in expression of GSTO1 between MDMA and saline treated rats in the western blot, suggesting the proteomic analysis used may not be suitable for the simple experimental plan utilized in this study. This study should be repeated with a more sophisticated experimental design, including larger numbers of animals in each group and different dosing regimens.

TITLE: A behavioural, neurochemical and proteomic analysis after treatment with 3,4-methylenedioxymethamphetamine and methamphetamine

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ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) administration leads to long term neurochemical and behavioural changes in rats and possibly humans. Proteomic analysis of MDMA's effects on the brain would appear attractive as a strategy to identify initial cellular effects but no such studies have been reported. We present such a study, using a dosing regimen and neurochemical and behavioural analyses previously validated within our laboratory. Methamphetamine, a commonly co-abused drug with similar pharmacology but differing neurochemical and behavioural effects, provided a positive control. It also indicated specificity of drug effects as well as information on combined effects relevant to human use. Rats were dosed with MDMA (10mg/kg), METH (10mg/kg) or the combination MDMA + METH (5+5mg/kg) twice a day for four days and sacrificed two weeks later when brains were removed for proteomic profiling and neurochemical analysis. Core temperature and locomotor activity was measured throughout the dosing periods. METH and MDMA + METH treatment led to acute hyperthermia. The combination treatment had a greater effect on increasing both core temperature and locomotor activity than each drug alone. The combination treatment also led to a decrease in cortical 5HT and 5HIAA concentrations. Ten proteins were identified which showed a change in abundance between at least two of the treatments, the biggest change being glutathione transferase omega-1. This could be significant as glutathione conjugates of MDMA have been associated with neurotoxicity. Other proteins showing changes are associated with important cellular functions and may indicate early mechanisms of drug induced effects on the brain.

INTRODUCTION

3,4-Methylenedioxymethamphetamine (MDMA) is a widely abused substance that has been shown to cause neurochemical and behavioural changes in animals (Clemens et al, 2005; Lyles and Cadet, 2003). These changes are most often reported as long term loss in markers of serotonin (5HT), and to a lesser extent other neurotransmitters. The use of MDMA has been rising around the world (United Nations, 2007), and there is evidence that humans may experience similar neurochemical changes as reported in animals after taking these drugs (McCann et al, 1998; Parrott, 2002). This possibility is supported by evidence from human recreational users of MDMA indicating they can achieve the same plasma concentrations of MDMA as in non-human primates exhibiting long lasting damage to serotonin terminals (Irvine et al, 2006; Mechan et al, 2006). However, mechanistic studies cannot be easily undertaken in humans, as they have usually been exposed to other drugs, such as methamphetamine, and the measurements that can be used are limited. There is also debate as to how to interpret the changes in markers used and how they relate to actual pathology (Baumann et al, 2007). A greater understanding of the cellular effects of these drugs is essential if we are to predict resulting pathologies at an early stage. This can only be achieved by experiments in animal models.

Animal studies have shown a link between MDMA administration leading to acute hyperthermia and long term changes in brain concentration of 5-HT and its transporters, as well as terminal degeneration (Battaglia et al, 1988; Camarasa et al, 2006). Malberg & Seiden (1998) gave rats MDMA at ambient temperatures ranging from 20-30°C. They showed a significant temperature dependent decrease in 5-HT concentrations in the cortex, hippocampus and striatum 2 weeks after administration. Other studies using multiple dosing regimens have also shown decreases in cortical 5HT and 5 hydroxyindole acetic acid (5HIAA) measured 3 and 30 days after drug treatment (Reneman *et al*, 2002c). These effects have also been demonstrated in monkeys (Mechan et al, 2006). Changes in

expression of specific proteins and genes have also been shown, such as changes in mRNA of various glutamate and 5HT receptors in different brain regions, including the cortex (Kindlundh-Hogberg et al, 2008; Kindlundh-Hogberg et al, 2006). Studies into the effect of MDMA on a broader range of proteins, which could precede these changes and perhaps act as biomarkers, or at least indicate initiating mechanisms of toxicity, have not been done.

Previous studies in our laboratory have established dose response relationships for MDMA and neurochemical and behavioural effects (Callaghan et al, 2006; Callaghan et al, 2007). Hippocampal SERT binding, 5HT concentration and synaptosomal 5HT uptake were reduced by 15 mg/kg MDMA but not 4 mg/kg given twice a day for four days (Callaghan et al, 2007). Rats treated with MDMA at 10 and 20 mg/kg showed significant decreases in cortical 5HT and 5HIAA two weeks after final administration (Callaghan et al, 2006). Doses ranging from 1 to 20 mg/kg MDMA have shown that increasing doses lead to dose dependent increases in core temperature and locomotor activity (Daws et al, 2000; Jaehne et al, 2007). Thus, dose related effects of MDMA on neurochemistry and behaviour are well established.

Methamphetamine (METH) has also been shown to lead to long term neurochemical and behavioural changes in animals and humans (Baicy and London, 2007; Marshall et al, 2007; Volkow et al, 2001), however, treatment with METH leads to different neurochemical changes and toxicity in the brains of rats compared to MDMA, with effects primarily on DA neurons, compared to 5HT neurons mostly effected by MDMA. Recent studies have demonstrated changes in protein expression in the brains of rats after METH treatment, using proteomic analysis (Iwazaki et al, 2006; 2007; 2008; Li et al, 2008; Liao et al, 2005). These studies used varying dosing regimens of METH and showed both up and down regulation of several proteins in the striatum, hippocampus, amygdala and cortex. Other evidence of damage to the brain caused by METH includes a study in which

rats were given four injections of METH, 5 mg/kg each injection. Striatal dopamine (DA) was shown to decrease 3 days after METH treatment, with a higher ambient temperature at the time of administration leading to greater decreases (Bowyer et al, 1994). It has also been shown that METH given at 4 mg/kg every four hours for a total of four injections can lead to degeneration of cortical neurons and damage to striatal DA and forebrain 5HT terminals (Marshall et al, 2007). These effects have also been demonstrated in monkeys (Yuan et al, 2006) and mice (Ali et al, 1994; Itzhak and Achat-Mendes, 2004).

MDMA and METH are widely used in human populations both separately and co-administered and have different long term effects on the brain. Accordingly, we have investigated the effects of these drugs on protein expression in the cortex of rats given previously described doses which are relevant to human exposure, using 2-dimensional difference in-gel electrophoresis (2D-DIGE), in combination with mass spectrometry (MS). Concurrent measures of thermoregulatory, behavioural and neurochemical effects of the drugs were also undertaken. METH acted as a positive control for MDMA to demonstrate drug selective effects but it is also of interest when one considers that it is the most commonly co-abused drug used with MDMA. Synaptosomal preparations were used as loss of terminal markers is one of the first and most robust indications of MDMA effects on the brain (Cadet et al, 2007). Most preclinical studies trying to understand the mechanisms of psychotropic drugs such as MDMA and METH on the brain has been done on rats. Mice, the favoured animal model for genomic and proteomic research, are not an appropriate model for MDMA research relevant to human health, as their neurochemical and behavioural responses to MDMA are distinctly different from rats, non-human primates and humans which are all similar (Lyles and Cadet, 2003; McCann et al, 1998; Mechan et al, 2006). The aim of this study was to determine whether there is a relationship between acute thermoregulatory effects, neurochemical changes and the proteomic profile of the cortex of rats administered MDMA and METH. To our knowledge this is the first

study to look at the effect of MDMA and MDMA + METH in combination on the proteomic profile of the rat cortex.

MATERIALS AND METHODS

Animals

16 Male Sprague-Dawley rats, weighing 300 ± 15 g at the start of experiments, were used for all testing. Before and after the testing period rats were housed in groups of 2-3, at an ambient temperature (T_A) of 21 ± 2°C and a 12h light/dark cycle (lights on at 08:00 h), with food and water available ad libitum. All experimentation was approved by the University of Adelaide Animal Ethics Committee and followed the Australian code of practice for the care and use of animals for scientific purposes.

Preparation and Administration of Drugs

(±)3,4-methylenedioxymethamphetamine HCl (MDMA) and (+)-methamphetamine HCl (METH) were obtained from The Australian Government Analytical Laboratories (Sydney, Australia). Treatment injections comprised of saline, MDMA (10 mg/kg), METH (10 mg/kg) or a MDMA + METH combination (5 mg/kg MDMA + 5 mg/kg METH). Drugs were dissolved in 0.9% saline administered at 1 ml/kg via i.p. injection. Controls received saline only. These doses and the dosing regimen were chosen based on previous studies and are consistent with those measured in humans using these drugs recreationally (Callaghan et al, 2006; Clemens et al, 2005; O'Shea et al, 1998).

Acute Effects of Drug Treatment

Rats were surgically implanted with telemetry devices (TA11CTA-F40, Data Sciences International, USA), which measure core body temperature (T_C), locomotor activity (LMA) and heart rate (HR), as reported previously (Bexis et al, 2004). The implants were placed

into the rats' abdominal cavity under anaesthesia (sodium pentobarbital, 60 mg/kg). At least 10 days recovery from surgery was allowed before rats underwent injection treatments. Radio receivers, placed underneath cages, received information from the implants at least hourly over the four treatment days, and transferred it to a computer which recorded the data using Dataquest LabPro software (Data Sciences International, USA).

The day before testing began rats were placed in individual cages in the testing room. Rats were assigned to one of four treatment groups, n = 4 per group (saline, MDMA, METH or MDMA + METH). Rats were dosed twice daily for four days at a T_A of 26°C. The dosing protocol was as follows: 08:00 h light and heater turned on; 09:00 h 1st daily drug administration; 17:00 h 2nd daily drug administration; 18:00 h heater turned off. The same protocol was repeated for four consecutive days. Two weeks after the final drug administration rats were anesthetized (sodium pentobarbital, 60 mg/kg), decapitated and their brains removed. Immediately after brains were removed, they were dissected in half and one half frozen for neurotransmitter analysis. The cortex of the other half was kept for immediate synaptosomal extraction for proteomic analysis.

Neurotransmitter Analysis

The cerebral cortex and striatum were dissected from the frozen brain and tissue samples were prepared for HPLC as described by Callaghan et al (2006). Concentrations of 5-HT, 5-HIAA, DA and dihydroxyphenyl acetic acid (DOPAC) were determined using a high performance liquid chromatography system with electrochemical detection (HPLC-EC) consisting of a CBM-20A with a working electrode housed at 30°C, potential of 0.7 V and a range of 50 nA. The mobile phase, delivered at a flow rate of 1 ml/min, consisted of 103 mM NaH_2PO_4, 0.5 mM octanesulphonic acid, 0.1 mM EDTA and 12.5% methanol, pH 3.8. Compounds of interest were separated using a 250 x 4.6 mm 5µ C18 column (Alltech, USA) and sampling was recorded using an LCsolution workstation (Shimadzu, Japan).

Statistical Analysis of Telemetry and Neurochemical Data

All calculations and analysis on LMA, T_C and neurotransmitter concentrations were done using Prism (version 5, Graph Pad, USA). The effects of LMA and T_C were separated into time periods where the most significant changes occurred, which for LMA was 0-2.5 and 2.5-8 hours after each injection, and for T_C was 0-2.5 hours after each injection. Mean data were calculated for each time period and analysed using 2-way ANOVA's, matching first by rows then columns, with Bonferroni's post hoc tests to compare between both day of treatment and drug administered. Cortical and striatal concentrations of 5HT, 5HIAA, DA and DOPAC were compared between drug treatments using 1-way ANOVA's with Tukey's post hoc test. All results are presented as mean ± SEM and *p*-value < 0.05 was taken as significant for all analyses.

Protein Sample Preparation

Cortical synaptosomes were isolated from brains of all rats using the Dunkley Percoll gradient method (Barber et al, 2007; Dunkley et al, 1988) with minor modifications. Once removed, cortices were minced in cold buffer containing 0.32 M sucrose and 1 mM EDTA (SED buffer; pH 7.4) at 9 ml SED for every 1 g of tissue. After washing in Krebs buffer, the pellet was then snap frozen in liquid nitrogen for storage.

Synaptosomal brain samples were defrosted and mechanically homogenized in 200 µL of IEF sample buffer containing 7 M Urea, 2 M Thiourea, 30 mM Tris, 4% CHAPS (TUC 4%) and incubated on ice for one hour. The samples were clarified by centrifugation at 20,000 x g at 4ºC for 30 minutes and 100µl of the protein-containing supernatants were collected into fresh sample tubes. Protein purification was performed using a 2D sample cleanup kit (Bio Rad, USA) according to the manufacturer's protocol with an additional

wash in 1 ml of cold acetone. The protein concentration was determined using an EZQ protein quantitation assay (Invitrogen, USA) against an ovalbumin standard curve.

Two-dimensional difference in-gel electrophoresis (2D DIGE)

Lyophilized DIGE Fluor minimal Cy2, Cy3 and Cy5 CyDyes (GE Healthcare, Uppsala, Sweden) were dissolved in anhydrous dimethylformamide at 200 pmol/μL, aliquoted into 1 μl volumes, and stored under argon at -80°C until required. When labelling, 100 μg of protein from each sample was added directly to the 1 μl (200 pmol) aliquot of either Cy3 or Cy5 dye. Dye swaps of biological replicates were performed to control for potential dye-associated bias. An internal standard was prepared by pooling 50 μg of protein from all 16 samples and labelled in bulk with 1,600 pmol of Cy2. Labelling reactions were performed on ice for 30 minutes in darkness and quenched with the addition of 1 μl of 10 mM lysine per 200 pmol of dye. Requisite Cy3, Cy5 and Cy2 samples were pooled and sufficient volumes of TUC4% were added to bring all eight samples to equivalent volumes (93 μl). Dithiothreitol (DTT: Sigma, St Louis, USA) and pH 3-11 NL IPG buffer (GE Healthcare) were also added such that the final samples contained 300 μg protein, 7 M urea, 2 M thiourea, 30 mM Tris, 4% w/v CHAPS, 10 mM DTT, 0.5% v/v ampholytes plus a trace amount of bromophenol blue for colour.

IPG strips (24 cm, pH 3-11, GE Healthcare) were rehydrated overnight in 450 μL of rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% 3-11 NL IPG buffer and 1.2% DeStreak reagent (GE Healthcare). Labelled sample mixtures were applied to the IPG strips by cup-loading. Isoelectric focusing was performed on an IPGphor II (GE Healthcare) at 20°C, with the current limited to 50 μA per strip for a total of 60,958 Vhrs. Strips were then equilibrated in 5 ml/strip of reducing buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT) then alkylating buffer (as above with 2.5% of iodoacetamide in place of DTT) for 15 minutes each. SDS-PAGE was carried

out using 12.5% polyacrylamide gels (25 cm x 20 cm x 0.1 cm) that were cast using the EttanDalt 12 gel casting system (GE Healthcare) according to the manufacturer's recommendations. Electrophoretic separation was performed using an EttanDalt 12 unit (GE Healthcare) in Tris-gly buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 15°C at a constant 105 V until the dye-front emerged from the bottom of the gel approximately 16 hours later.

Three preparative 2D gels were prepared as described above with 300 μg of unlabeled protein from the saline, METH, MDMA and MDMA + METH treatment groups. Prior to casting the glass plates were treated with 4 mL of Bind silane solution (80% ethanol, 2% acetic acid, 0.1% Bind silane; GE Healthcare). The resulting gels were stained with LavaPurple (Gel Company, Tuebingen, Germany) according to the manufacturer's recommendation.

Gel imaging and image analysis

All gels (analytical and preparative) were scanned using the Ettan DIGE Imager (GE Healthcare) at 100 µm resolution. Image analysis was undertaken using DeCyder 2D software (version 6.5, GE Healthcare). Each analytical gel image was processed separately in the Differential In-gel Analysis (DIA) module of DeCyder prior to export to the Biological Variation Analysis (BVA) module. In DIA, spot detection based on an estimated 5,000 spots, background subtraction and in-gel normalization processes were carried out automatically. Exclusion filters were set to reject spots with slopes > 1.1, areas < 600, volumes < 10,000 and a peak heights < 80 and > 65,000. Regions of the gels that showed poorly resolved spot patterns (e.g. at the edges or areas affected by vertical streaking) were manually excluded. The DIA workspaces were imported into BVA for spot matching and comparative analysis. Errors in the automatically assigned spot matches were re-matched manually. In order for a matched spot to be considered as statistically

significant for a given comparison it was required to have a *p*-value < 0.05 as determined by an unpaired, two-tailed Student's t-test that was performed in the BVA module of DeCyder, and demonstrate a fold-change greater than the effect size determined by a post-hoc power calculation. This was performed using the Java applet Piface (version 1.64, available at http://www.cs.uiowa.edu/~rlenth/Power/) (Lenth, 2007). Briefly, the normalized volumes of the matched spots were exported from DeCyder into Excel (Microsoft, USA) using the XML Toolbox function. Standardized abundance (SA) values were determined by dividing the spot volumes of the Cy3 and Cy5 channels by the Cy2 channel for each of the eight gels. The SA values were $\log_{10}$ transformed to normally distribute the data and the standard deviation of each spot was calculated. The 75% percentile value of the standard deviation was used as the measure of variance in the power calculation (Karp and Lilley, 2005). Other parameters required for the calculation were the number of biological replicates (n=4 for each group), the significance level (0.05), and the level of power (80%). The resulting effect sizes were exponentiated to reverse the $\log_{10}$ transformation, thereby providing the minimum fold-change at which the mean SA values of the compared groups could be considered significantly different. Accordingly, spots with *p*-values < 0.05 and fold-changes < -1.4 and > +1.4 were identified as spots-of-interest. Spot detection of preparative gels was also performed in the DIA module prior to being exported into the BVA workspace as "pick gels". Only the spots classified as spots-of-interest were manually matched from master gel to the pick gels. Pick lists for each preparative gel were created in DeCyder

Mass Spectrometry

Spots-of-interest were excised in an automated fashion using an Ettan™ Spot Picker robot (GE Healthcare) based on the pick lists. Excised gel pieces were digested overnight at 37°C with trypsin (100 ng of sequencing grade modified trypsin [Promega, USA] in 20 μL

of 5 mM ammonium bicarbonate, 10% acetonitrile [ACN]). The extracted tryptic peptides were separated using an 1100 HPLC system equipped with a Protein ID Chip column assembly (40 nL trap column with 0.075 x 43 mm C-18 analytical column) housed in an HPLC-Chip Cube (all Agilent Technologies, Santa Clara, USA). This was interfaced directly with an HCT Ultra 3D-Ion-Trap mass spectrometer (Bruker Daltonics, Germany) operating in positive ion mode. The column was equilibrated with 0.1% formic acid (FA), 3% ACN at 0.5 µl/min and the samples eluted with an ACN gradient of 3%-31% over 32 minutes. Ionisable species ($300 < m/z < 1,200$) were trapped and one or two of the most intense ions eluting at the time were fragmented by collision-induced dissociation. Active exclusion was used to exclude a precursor ion for 30 seconds following the acquisition of two spectra. MS and MS/MS spectra were subjected to peak detection and de-convolution using DataAnalysis (version 3.4, Bruker Daltonics). The MS and MS/MS mass lists were exported into BioTools (version 3.1, Bruker Daltonics) then submitted to Mascot (version 2.2). The specifications were: taxonomy: mammalia, database: Swissprot 56.4, enzyme: trypsin, fixed modifications: carbamidomethyl of cys, variable modifications: oxidation of met, mass tolerance: 0.3 Da for MS and 0.4 Da for MS/MS, peptide charge: 1+, 2+ and 3+ and missed cleavages: 1.

Western Blotting

Protein samples from saline and MDMA treated rats, which showed the biggest difference in GSTO1 expression, were re-suspended in sample buffer comprised of 2% SDS, 10% glycerol, 62.5 mM TrisHCl pH 6.8, Halt protease inhibitors (Sigma), and 65 mM DTT. Fifty µg of each sample plus 2 µl of molecular weight standards (Dual Colour Precision Plus, BioRad) were loaded onto 10% polyacrylamide gels and electrophoresed at 200 V. Protein was transferred using a semi-dry apparatus for 2 hours at 70 mA per blot onto low fluorescent PVDF membrane. Membranes were blocked in 5% skim milk PBST and

incubated overnight at 4ºC with the primary antibodies diluted in 1% skim milk PBST. Membranes were thoroughly washed with PBST and incubated in the dark at room temperature for 1.5 hours with ECL Plex fluorescent detection Cy3 antibody (GE Healthcare) at a 1:1000 dilution. GSTO1/2 (FL-241), rabbit polyclonal IgG (Santa Cruz Biotechnology) was used at 1:200. Beta actin loading control (mouse) was used at 1:500. Membranes were washed with PBST, followed by washes with PBS, air dried, and imaged using a Typhoon 9400 Imager (GE Healthcare). The primary GSTO1/2 antibody and beta actin levels were detected using fluorescent Cy3 and Cy5 antibodies, and quantified using Image Quant V5 software. Levels of GSTO1/2 expression were normalised to beta actin expression in each sample.

RESULTS

Effect of Treatment on Locomotor Activity and Core Temperature (Figure 1)

MDMA and METH treatment both led to increases in LMA in the 2.5 hours following the 1st injection of drug. MDMA treated rats had a significantly higher LMA than saline treated rats after the 1st injection on day 2 only ($p < 0.05$), while on day 1 METH treated rats had a significantly higher LMA than saline rats after both the 1st ($p < 0.001$) and 2nd ($p < 0.01$) daily injections. MDMA + METH treatment had no significant effect on LMA in the 1st 2.5 hours after each injection, but rats did increase their LMA during the period after this, when LMA of rats given the other treatments had decreased. LMA of MDMA + METH treated rats was higher than saline after the 1st injection of day 2 ($p < 0.05$), 3 ($p < 0.01$) and 4 ($p < 0.001$) as well as after the 2nd injection on day 4 ($p < 0.05$) during the time period 2.5 - 8 hours after injection.

MDMA treatment led to an apparent decrease in T_C, with T_C after the 1st injection on day 2 less than after saline treatment on the same day ($p < 0.05$). Administration of METH led to an increase in T_C, which was significantly higher than saline after the 1st injection only ($p <$

0.05). MDMA + METH treatment led to increased T_C compared to saline after the 1st daily injection on day 1 ($p < 0.01$) and 2 ($p < 0.01$) only, and the 2nd daily injection on day 1 ($p < 0.001$), 2 ($p < 0.001$) and 3 ($p < 0.01$) only.

The pharmacodynamic effects of these drugs on thermoregulation and locomotor activity indicate that the drugs were administered at appropriate doses to modify behaviour.

Brain Concentrations of Neurotransmitters (Figure 2)

MDMA + METH treatment led to significant decreases in 5HT ($p < 0.05$) and 5HIAA ($p < 0.01$) concentration in the cortex compared to saline treated controls. There were no significant differences in DA or DOPAC in the cortex, and no significant changes compared to saline in any of the neurotransmitters measured in the striatum. These changes indicate that the cortex is a major target for the neurochemical effects of the drugs.

Protein Expression Changes in Cortex

Figure 3 shows a typical 2-DE gel pattern of protein extract from the cortex. A pair-wise comparison of each group performed against the others using a two-tailed Student's T-test identified 10 spots-of-interest with fold-changes > 1.4 in magnitude (highlighted in Figure 3). Protein identities were obtained for seven of the ten spots by mass spectrometry. Characteristics of these proteins with respect to their appearance in specific comparisons are summarized in Table 1. Table 1 includes the compared treatment groups and observed fold-changes in spot abundance, the spot numbers, the identified protein(s) and SwissProt accession numbers, the combined ion scores and cut-off score for an individual ion whereby the combined ion score is the sum of the best scores for each individual peptide (excluding redundant peptide hits), the number of unique peptides matched via MS/MS spectra; the percentage sequence coverage, and the exponentially modified protein abundance index (emPAI value) as an approximate measure of relative quantitation

(Ishihama *et al*, 2005). The observed molecular weight (MW) and pI values presented in Table 1 were estimated according to the positions of the spots on the gel. The predicted values of MW and pI were obtained from SwissProt and included known fixed modifications of the identified proteins such as S-amidomethylation and *N*-terminal processing (e.g. acetylation or removal of signal sequence where indicated), however did not include other co- or post-translational modifications. Descriptions of the subcellular localization and molecular function of each protein were also obtained from SwissProt.

Multiple proteins were identified in spots 452 and 459, however alpha-centractin protein appears to be the most abundant protein in both spots based on the significantly larger emPAI scores. Accordingly, it is likely that this protein is responsible for the changes in abundance observed for this spot between the treatment groups. The identification of dual specificity mitogen-activated protein kinase kinase 1 as the sole component of spot 779 could be described as tentative based on the fact that only one peptide for this protein was identified. Relative to many other spots, the three spots that could not be identified (spots 864, 1136 and 1158) were faint and presumably contained insufficient amounts of protein for successful MS analysis even on preparative gels (Figure 3).

Western Blot Confirmation of GSTO1 Protein

The relative of abundance, normalised to β-actin, of glutathione transferase omega-1 was not increased after MDMA administration compared to saline treated controls (Fig. 4).

DISCUSSION

The core temperature, behavioural and neurochemical effects of the drugs were generally consistent with our previous results and those of other laboratories (Clemens *et al*, 2004; Daws et al, 2000; Jaehne et al, 2007). Interestingly, there were also drug-selective changes

in synaptic proteins which may reflect early mechanisms of drug induced neuronal damage.

All drug treatments resulted in an increase in LMA indicating the drugs had been administered at psychoactive doses. METH and MDMA + METH treatment led to increases in T_C, and rats showed tolerance to this effect over the four treatment days. Studies using varying dosing regimens have shown tolerance, sensitization or no change to the hyperthermic effects of these drugs after repeated exposure (Clemens et al, 2007; O'Shea et al, 1998). The effects on T_C are important to measure as this variable has been shown to influence the neurochemical outcome of treatment with these drugs (Malberg and Seiden, 1998).

Rats treated with the combination treatment MDMA + METH showed significant decreases in cortical concentrations of 5HT and 5HIAA, and also had significantly lower striatal concentrations of 5HT than METH treated rats two weeks after drug treatment. This combination has also been shown to lead to depletions of 5HT and 5HIAA in the cortex for longer periods (Clemens et al, 2007; Clemens et al, 2004). This is in contrast to MDMA treated rats, which in our current study showed no decreases in 5HT or 5HIAA in either brain region measured. This is consistent with other papers that have shown hyperthermia is required to see significant changes in neurotransmitter concentration after single doses (Malberg et al, 1996; Malberg and Seiden, 1998). We also showed no significant changes in 5HT or 5HIAA concentrations in the brains of METH treated rats. Previous studies suggest that more frequent dosing is required to see long term changes in these neurotransmitters (Bowyer et al, 1994; Broening et al, 2005; Clemens et al, 2007; Clemens et al, 2004).

No significant changes in DA or DOPAC were shown after any treatment in either the cortex or striatum. It was expected that changes in the METH and MDMA + METH treated groups would occur, based on previous results in other laboratories (Bowyer et al,

1992; Clemens et al, 2004). However, there was more variability in DA and DOPAC concentrations than 5HT and 5HIAA concentrations which may explain the lack of significance. A trend towards a decrease in DA was shown in the cortex of rats treated with METH or MDMA + METH.

While there were clear differences in the effects of the treatments used in parameters which have been measured previously, the combination treatment having the greatest effect all around, the proteomic analysis is not as clear. Looking at changes in concentrations of 5HT, DA and their metabolites is useful in relating damage to the brain to behavioural changes in animals, but more subtle changes in proteins may be able to be used as earlier biomarkers for damage occurring in the brain. In these initial proteomic studies we concentrated on the cortex as this area has been shown in rats (Malberg and Seiden, 1998; Marshall et al, 2007), non-human primates (Mechan et al, 2006) and indirectly in humans (McCann et al, 1998) to demonstrate long term changes after MDMA or METH treatment. Disrupted cortical function has also been suggested to be associated with psychological and cognitive deficits related to MDMA use (Quednow *et al*, 2006a). We looked at a synaptosomal preparation to improve resolution of proteins, and because many of the changes reported in animals after MDMA or METH have involved synaptosomal markers, such as decreased transporter numbers or function and degeneration of terminals (Callaghan et al, 2007; Marshall et al, 2007; Tata et al, 2007).

Analysis of the 2D-gels showed a number of changes in protein expression between treatment groups, although most of these changes were only moderate. This was not surprising, as we anticipated a proteomic analysis would be more useful for identifying only initial changes occurring prior to more the dramatic signs of neurotoxicity. While the thermoregulatory and neurochemical effects were greatest compared to saline in the

MDMA + METH group, most of the protein changes occurred in MDMA or METH treated rats, suggesting there was no clear relationship between the previously measured parameters and the newly studied proteomic changes. However, several identified proteins were of interest because of their function and roles in disease. These included aconitate hydratase, mitochondrial (ACON), which is involved in breakdown of citrate and was up-regulated in the METH group, ubiquitin-conjugating enzyme E2 variant 1 (UB2V1), a novel regulator of protein ubiquitination which was down-regulated in the MDMA group, and dual specificity mitogen-activated protein kinase 1 (MEK1), which is involved in signal transduction and showed changes in both the MDMA and METH groups. Also showing changes were glutathione transferase omega-1 (GSTO1), which was up-regulated after MDMA treatment, and showed the biggest change in expression compared to saline treated controls. There were also differences between the MDMA + METH group and the other two drug treatment groups including ATP synthase B chain, mitochondrial precursor, which is involved in cell respiration, as well as the proteins mentioned above MEK1, ACON, and UB2V1.

GSTO1, which was identified as showing the biggest difference in expression between two treatment groups using 2D-DIGE, is a member of the glutathione transferase super family (Board and Anders, 2007). The gene for GSTO1 has recently been linked to the age of onset of Alzheimer's and Parkinson's diseases (Li et al, 2003). It has been suggested that GSTO1 has an effect on the post-translational processing of interleukin 1-beta, and is a target for some cytokine release inhibitory drugs (Laliberte et al, 2003), which may contribute to its association with neurodegenerative diseases. It is also important to note that glutathione conjugates of the catechol metabolites of MDMA have been shown to be neurotoxic when injected into the brain (Monks et al, 2004), and have recently been found in human users. There was also two mitochondrial proteins affected, which is also of

interest as mitochondrial dysfunction appears to play a role in neurotoxicity induced by both MDMA and METH (Quinton and Yamamoto, 2006).

Other proteins that were identified as having an altered abundance after drug treatment have also been shown to be associated with neurodegenerative and other neurological diseases. For example, MEK1 has been shown to be involved in focal cerebral ischemia (Henriksson et al, 2007), nuclear localization of it has been associated with Alzheimer's disease (Zhu et al, 2003), and its activation has been shown to be induced by opioid receptor agonists (Asensio et al, 2006). Other proteins that showed differences in abundance between treatment groups including glutamine synthase, adenosylhomocysteinase, aconitate hydratase and ATP synthase B chain, mitochondrial precursor, have all been shown to have some association with neurological diseases such Alzheimer's, Parkinson's, epilepsy and schizophrenia.

Some of the proteins identified in the current study overlap with those found in previous studies looking at the effect of METH on proteomic profiling, even though these studies used different dosing regimens and brain regions, and the changes shown here were not all induced by METH. Iwazaki et al (2008) showed down-regulation of glutamine synthetase and aconitate hydratase in the amygdala of rats administered 1 mg/kg METH four hours earlier. This study, as well as another study done recently on the effect of METH on proteomic profile in rats, also showed changes in proteins that are not the same, but are closely related to the proteins we have shown changes in. These include ubiquitin conjugating enzyme E2N (Iwazaki et al, 2008; Li et al, 2008), ATP synthase D chain, mitochondrial precursor (Iwazaki et al, 2008; Li et al, 2008), and other glutathione S-transferase proteins (Iwazaki et al, 2008).

As the protein GSTO1 showed the greatest changes between any two groups, and because it appears to be an interesting protein which may show a relationship with MDMA after further investigation, we attempted to confirm the changes seen using western blot

analysis. Unfortunately expression of this protein using western blot was variable, so differences between treatment groups did not confirm with 2D-DIGE results. This indicates that further method development is required to improve the quantitative assessment of small changes in protein expression in this type of experiment so that more useful results can be achieved.

In conclusion, we have shown changes in LMA, T_C and cortical and striatal neurotransmitter concentrations in rats administered MDMA, METH or a combination of the two at doses which are relevant to human exposure. We have also used 2D-DIGE to analyse the protein expression profile in the cortex of these rats and shown changes in a number of proteins associated with several important brain functions, as well as several associated with degenerative disorders such as Alzheimer's and Parkinson's disease. There does not seem to be a clear relationship between the proteomic profile and the pharmacodynamic profile of these drugs. More comprehensive studies using a range of doses, dosing regimens, brain regions and time from last administration to removal of brain are warranted to clarify the relationship. This approach may be a good way to study the effects of psychotropic drugs in a rat model, and could lead to the identification of biomarkers or drug targets, which would aid in early prediction or treatment of MDMA and/or METH adverse effects on brain function before the development of severe psychological and neurological impairment.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that, except for income received from the primary employer, no financial support or compensation has been received from any individual or corporate entity for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Miss Jaehne had a major input in the experimental design, conducted all telemetric studies and neurochemical analyses, statistical analysis and graphical presentation of this data, and prepared the manuscript for submission.

Dr Colella was involved in the experimental design, conducted all proteomic procedures and contributed to the interpretation of the data collected and preparation of the manuscript, including writing much of the proteomics sections of the Methods and Results.

Dr Penno conducted statistical analysis of proteomic results and contributed to the interpretation of the data collected and preparation of the manuscript, including writing much of the proteomics sections of the Methods and Results.

Dr Hoffmann was involved in the experimental design, and contributed to the interpretation of the data collected and preparation of the manuscript.

Associate Professor Irvine was involved in the experimental design, and contributed to the interpretation of the data collected and preparation of the manuscript.

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TABLES

Spot	Drug	Fold Change	Protein	Accession	Ion score/ cut off	No. unique peptides	% Seq. coverage	emPAI	Predicted MW(kDa), pI	Observed MW(kDa), pI	Celular Location	Function
Protein changes compared with saline control rats												
856	MDMA	3.32	Glutathione S-transferase omega-1	GSTO1_RAT	386/34	8	34.4	1.46	27.9, 6.3	30, 6.3	Cytoplasm	Glutathione-dependent thiol transferase
452	MDMA	-1.62	Alpha-centractin	ACTZ_RAT	505/34	9	40.4	0.81	42.7, 6.2		Cytoplasm	Component of dynactin complex involved in mitosis, nuclear migration & general cellular traffiking
			Glutamine synthetase	GLNA_RAT	120/34	2	9.7	0.16	43, 6.7		Cytoplasm	Catalyses conversion of glutamate to glutamine
			Dual specificity mitogen-activated protein kinase 1	MP2K1_RAT	98/34	2	12.2	0.16	43.8, 6.2	48, 6.2	Mitochondrion inner membrane	Transfers electorns in mitochondrial respiratory chain; activates ERK MAP kinases
			COP9 signalosome complex subunit 3	CSN3_RAT	64/34	1	3.1	0.07	48.4, 6.2		Cytoplasm, Nucleus	Part of complex involved in various cellular and develpomental processes
1191	MDMA	-1.67	Ubiquitin-conjugating enzyme E2 variant 1	UB2V1_MOUSE	166/35	4	30.6	1.55	16.5, 9.1	15, 6.4	Nucleus	Novel regulator of protein ubiquitination
459	MDMA	1.43	Alpha centractin	ACTZ_RAT	635/34	13	48.8	2.06	42.7, 6.2		Cytoplasm	Component of dynactin complex involved in mitosis, nuclear migration & general cellular traffiking
	METH	1.64	Dual specificity mitogen-activated protein kinase 1	MP2K1_RAT	566/34	10	29.5	1.07	43.8, 6.2	48, 5.9	Mitochondrion inner membrane	Transfers electorns in mitochondrial respiratory chain; activates ERK MAP kinases
			Adenosylhomocysteinase	SAHH_RAT	326/34	7	18.1	0.49	48, 6.1		Cytoplasm, Melanosome	Plays role in control of methylations
			Septin-2	SEPT2_RAT	72/34	2	7.8	0.08	41.7, 6.1		Cytoskeleton	Involved in cytokinesis
864	METH	-2.53	–	–	–	–	–	–	–	26, 6.1	–	–
985	METH	1.41	Aconitate hydratase, mitochondrial	ACON_RAT	107/35	2	5.1	0.08	86.1, 8.7	25, 8.0	Mitochondrion	Catalyses reaction of citrate to isocitrate
1136	MDMA +METH	-1.64	–	–	–	–	–	–	–	18, 8.2	–	–
Protein changes compared with METH treated rats												
779	MDMA	-1.57	Dual specifity mitogen-activated protein kinase 1	MP2K1_RAT	104/34	1	5.6	0.08	43.8, 6.2	32, 7.4	Mitochondrion inner membrane	Transfers electorns in mitochondrial respiratory chain; activates ERK MAP kinases
	MDMA + METH	-165										
985	MDMA + METH	-1.75	Aconitate hydratase, mitochondrial	ACON_RAT	107/35	2	5.1	0.08	86.1, 8.7	25, 8.0	Mitochondrion	Catalyses reaction of citrate to isocitrate
1014	MDMA + METH	-1.41	ATP synthase subunit b, mitochondrial	AT5F1_RAT	63/35	2	5.1		29, 9.9	24, 8.0	Mitochondrion inner membrane	Produces ATP from ADP in presence of photon gradient
1158	MDMA + METH	1.59	–	–	–	–	–	–	–	16, 6.2	–	–
Protein changes compared with MDMA treated rats												
1191	MDMA + METH	-1.48	Ubiquitin-conjugating enzyme E2 variant 1	UB2V1_MOUSE	166/35	4	30.6	1.55	16.5, 9.1	15, 6.4	Nucleus	Novel regulator of protein ubiqutination

Table 1. Summary of identified proteins that exhibited change in abundance after various drug treatments

FIGURE LEGENDS

Figure 1. Mean locomotor activity (LMA) of rats measured for 8 hours (a) and mean core temperature (T_C) of rats measured for 2 ½ hours (b) after each of two daily injections (09:00 and 17:00) of MDMA (10 mg/kg), METH (10 mg/kg) or MDMA + METH (5 + 5 mg/kg) for four consecutive days. All data represent mean ± SEM (n = 4 per group). Data were analysed using 2-way ANOVA's (matching by rows and columns separately) with Bonferroni post hoc tests. * Represents significant difference compared to day 1 of the same drug treatment, # significant difference c.f. day 2 and + significant difference c.f. day 3. ^ Represents significant difference to saline treatment on the same day, $ significant difference to MDMA and ~ significant difference to METH. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2. Cortical and striatal concentrations of 5HT, 5HIAA, DA and DOPAC in rats measured two weeks after rats were administered two daily injections (09:00 and 17:00) of MDMA (10 mg/kg), METH (10 mg/kg) or MDMA + METH (5 + 5 mg/kg) for four consecutive days. All data represent mean ± SEM (n = 4 per group). Data were analysed using 1-way ANOVA's with Tukey's post hoc tests. ^ Represents significant difference to saline treatment, $ significant difference to MDMA and ~ significant difference to METH. ^ $P < 0.05$, ^^ $P < 0.01$, ^^^ $P < 0.001$.

Figure 3. (a) A representative 2-DE gel image of expressed proteins in rat cortex. The positions of spots that showed differences in protein abundance between two or more treatment groups are marked with corresponding spot numbers as shown in Table 1. (b) 3-D representation of relative levels for spot 856, which was identified as GSTO1. (c) Depicted change in relative protein abundance calculated from standardized abundance values for spot 856.

Figure 4. Western blot analysis of glutathione transferase omega-1 in the cortex of the rat. No differences were shown between rats treated with MDMA compared to saline controls.
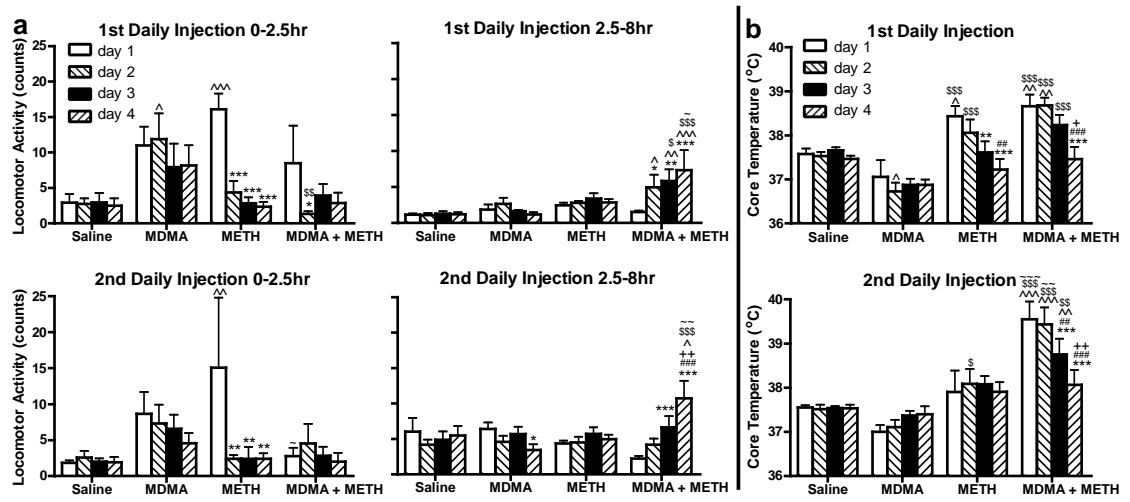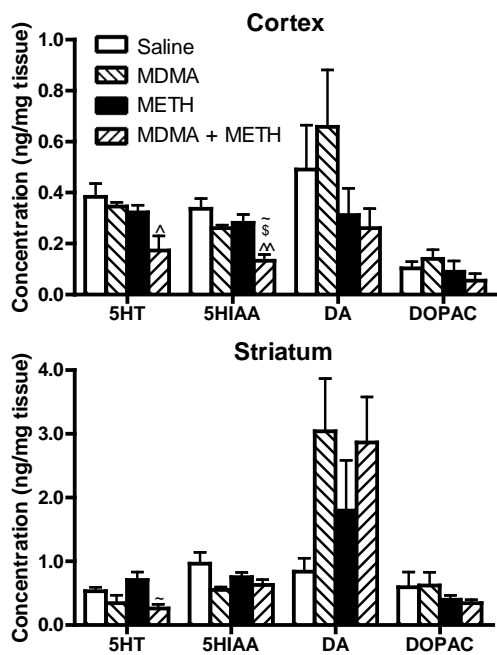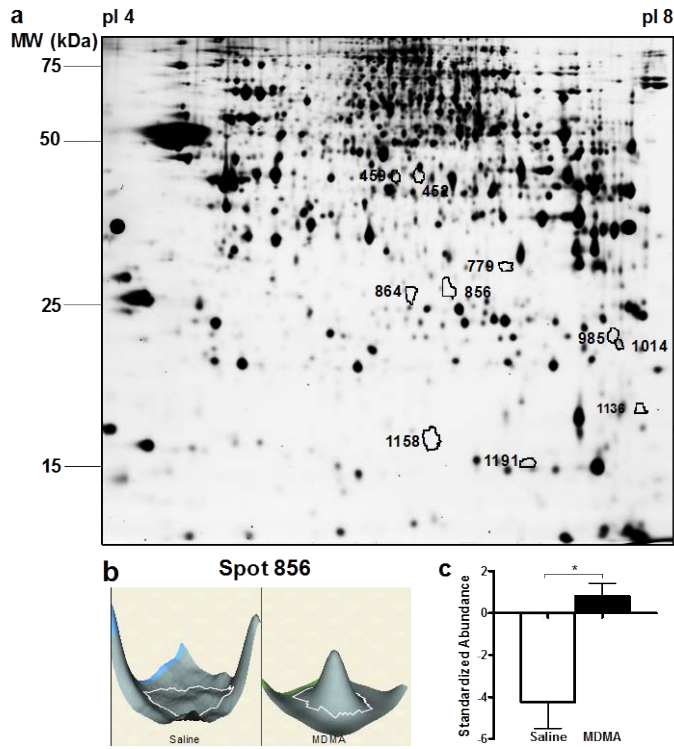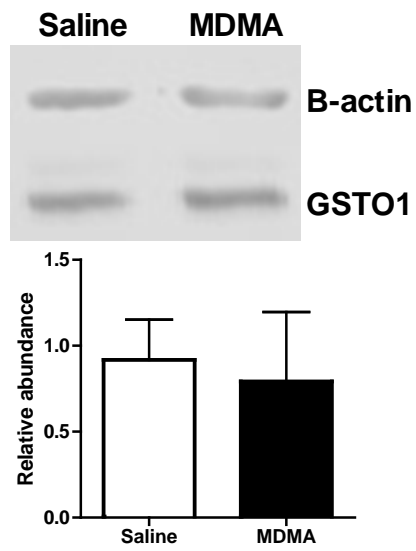
FIGURES

Figure 1

Figure 2

Figure 3

Figure 4

6.   Discussion

The adverse effects of stimulant drugs on hyperthermia and neurotoxicity are very important in the overall health of stimulant users. The use of stimulant drugs, especially MDMA, can lead to the development of several different health problems. When MDMA is taken it can lead to acute increases in T_C, particularly when taken at high ambient temperature, directly leading to symptoms such as cardiac arrhythmias, acute renal failure, rhabdomyolysis and disseminated intravascular coagulation, which can in turn lead to death in some cases (Gowing *et al*, 2002; Williamson *et al*, 1997). Stimulant drugs, including MDMA, can also increase HR and blood pressure which may also lead to various clinical problems (Badon *et al*, 2002). In addition to the acute effects, MDMA is also associated with long term neurological changes in animal studies, such as decreases in concentration of 5HT and its transporters, and loss of 5HT neurons (Itzhak and Achat-Mendes, 2004; Malberg and Seiden, 1998). The cardiovascular and neurological pathologies shown following MDMA administration are exacerbated by increased T_C at time of drug taking, suggesting it is very important to get a better understanding of the effects of MDMA on thermoregulation. It has also been shown that high ambient temperature may enhance the rewarding effects of MDMA in rats and humans (Cornish *et al*, 2003; Parrott, 2002; 2006), which in turn may influence patterns and levels of use. These effects are very unpredictable in human users, with both experienced and inexperienced users suffering hyperthermia after taking a large range of doses.

In order to investigate these issues we examined the literature and found that at the time of starting work for this thesis, there were no reasonable scientific explanations for why these effects are so unpredictable in humans. We therefore decided to study an aspect of thermoregulation which had not previously been studied with respect to MDMA or other stimulant drugs, that is, behavioural thermoregulation. We also adopted telemetry for

our T_C measurement, not only because of the ability to record continuously, but also because previous studies using rectal probes have been criticised as causing artifactual changes in T_C due to stress associated with animal handling. As a first step in this approach we looked at the dose-response relationship of four stimulant drugs, MDMA, PMA, methamphetamine and cocaine, to increase T_C at a high ambient temperature, and then observed the subsequent behavioural thermoregulation in a thermal gradient. This was important as their comparative potencies on thermoregulation could not be assessed from previous studies which had only used limited doses. It was also of importance in relating to human use of these drugs which are often taken in combination. These experiments established how the rats behaved in our set up, and gave us optimal doses to use in subsequent experiments (Jaehne *et al*, 2007). The second study extended these findings to look at the effect of chronic dosing on both behavioural and autonomic thermoregulation (Jaehne *et al*, 2008). This simulated the more regular weekly use of these drugs which is the most common pattern reported in humans. The third study compared the effects of MDMA between SD rats, and a rat model of depression, the FSL rat. This study was prompted by the association between drug use and depression, which has been reported in MDMA users (Green *et al*, 2003; Guillot and Greenway, 2006; Lieb *et al*, 2002; Soar *et al*, 2001) and the cellular effects of MDMA which targets 5HT systems which are known to be important in the development of depression (Dremencov *et al*, 2005; Green *et al*, 2003; McCann *et al*, 1998). Due to the demonstrated long term effects of MDMA on the brain, we also looked at changes in neurotransmitter concentration in rats in these experiments. An initial proteomics investigation was undertaken in the fourth study presented in this thesis. This latter study was prompted by the need to identify early effects of MDMA and methamphetamine which may indicate the latter development of neurotransmitter loss and neuronal damage. The main goal of this thesis was therefore to explore possible

explanations for the effects of MDMA on thermoregulation, as well as to investigate the possible longer term consequences of MDMA induced hyperthermia on the brain.

To make the discussion of the results simple to follow, it has been divided into different sections, reflecting the different types of behavioural and physiological parameters measured. The first aspect which will be discussed will be the thermoregulatory results from rats confined to the set ambient temperatures, then the thermoregulatory results of rats in the thermal gradient. After that, the more physiological results including cardiovascular and locomotor activity changes will be discussed, and finally the effects of MDMA and the other drugs tested on the brain will be covered.

Core Temperature at a Constrained Ambient Temperature

We have shown in each of the first three papers that doses of MDMA ranging from 7.5 to 10 mg/kg lead to T_C increases of around 4ºC, when MDMA is given to SD rats placed in an ambient temperature of 30ºC for 30 minutes. This is higher than reported in other studies where MDMA was given at similar doses and ambient temperatures (Clemens *et al*, 2007; Malberg and Seiden, 1998; Mechan *et al*, 2002). Changes in T_C were also higher in saline treated control animals in our experiments. Environmental factors may explain this, as our animals were held in a relatively confined space while at the high ambient temperature. Although restraining rats can lead to increases in T_C (Gordon, 1990), this may be more relevant when trying to translate these data to humans in a club environment where often there is limited space for individuals in a warm ambient temperature (Parrott *et al*, 2006).

Throughout this thesis we have shown that there are differences between the hyperthermia inducing effects of MDMA compared to other stimulant drugs. MDMA, PMA and methamphetamine all showed a similar increase in T_C after a 40 μmol/kg dose (9.19, 8.08 and 7.44 mg/kg respectively), but lower doses had varying effects. The

increasing rank order of potencies of the drugs we tested was cocaine < MDMA = PMA < methamphetamine. If these potencies were to translate to humans, then this factor alone could not explain the differences in the number of deaths reported in the literature involving hyperthermia. Our assessment of the literature over the last 20 years has shown that methamphetamine has led to the lowest number of deaths involving hyperthermia, followed by cocaine, then the 'ecstasy' type drugs MDMA and PMA. However the shape, or slope, of the dose response curve may be of more importance. We showed that MDMA and PMA had steeper slopes than methamphetamine and cocaine. When this is considered in combination with the report of De la Torre *et al* (2000) showing non-linear pharmacokinetics of MDMA in humans, and a recent report showing similar effects in rats (Baumann *et al*, 2009), these results may relate to some of the case reports of severe hyperthermia. These findings indicate, in our animal model at least, that additional administrations of MDMA and PMA could result in a very large, possibly dangerous, increase in T_C. This would be less likely to occur when taking methamphetamine or cocaine. These complex dose response relationships may underline the difficulties in predicting adverse outcomes from administrations of these drugs in human users.

The study presented here investigating the long term effects of MDMA showed little effect on changing the thermoregulatory response of SD rats in either high or normal ambient temperature after repeated administrations, suggesting no long term tolerance to the effect on T_C developed. However, a difference between dosing days 1, 2 and 3 of each week was shown. That is, on the first day of each week the change in T_C was higher than on the second and third days suggesting a more acute type of tolerance. Other studies have demonstrated conflicting results when measuring tolerance to MDMA induced hyperthermia. Clemens *et al* (2007) showed hyperthermia after the first weekly administration of 8 mg/kg MDMA to female Albino Wistar rats at 28°C but not after the 8th or 16th. O'Shea *et al* (1998), as in our present study, reported no change in MDMA

induced changes in T_C after giving male Dark Agouti rats 4 mg/kg twice a week for eight weeks. Other studies have also had mixed responses (Green *et al*, 2005; Mechan *et al*, 2001; Shankaran and Gudelsky, 1999). Thus the issue of tolerance and/or sensitization is unresolved and it is unclear whether this could contribute to the unpredictable hyperthermia reported in human users. This is important as humans show tolerance to many drugs. However, in some cases tolerance only develops to some effects of the drugs so increasing the dose to get the same desired psychotropic effect may lead to dangerous consequences if tolerance does not occur to hyperthermia for example.

There are several links between MDMA use and depression in human users. On one side, long term depletion of 5HT has been shown after MDMA-induced hyperthermia (Malberg and Seiden, 1998; McCann *et al*, 1998), which had led to the hypothesis that use of MDMA may lead to long term depression and mood disturbance (Green *et al*, 2003). Contrasting this, several studies have also shown that depression may precede MDMA use in some users (Guillot and Greenway, 2006; Lieb *et al*, 2002; Soar *et al*, 2001). Whether ecstasy use causes depression or not, it is clear that many people who take ecstasy also have depression. Understanding the cause/effect relationships associated with MDMA use and depression is very difficult to examine in humans and we speculated that an animal model may well provide useful insights. We therefore compared the effects of MDMA in a rat model of depression, the FSL rat, and compared it with our standard SD strain. We showed that when MDMA was administered at a high ambient temperature, T_C rose to a much higher level in FSL rats compared to SD's. This may be partially explained by T_C at the start of confinement to high ambient temperature, being a little higher (1ºC) in this strain, possibly due to stress (Gordon, 1990; Irvine *et al*, 1997; McDougall *et al*, 2005; Zambello *et al*, 2008). However, the change in T_C after both doses of drug was also greater in FSL rats and MDMA led to a higher rate of fatalities due to hyperthermia in FSL rats. These data suggest increased sensitivity to adverse MDMA effects in an animal model of

depression. FSL rats have been shown to be more sensitive to the hypothermic effects of cholinergic (Overstreet *et al*, 1998), dopaminergic (Crocker and Overstreet, 1991), and serotonergic (Wallis *et al*, 1988) drugs. These results, and others showing the effect on behaviour (Shayit *et al*, 2003), suggest that FSL rats are more sensitive to serotonergic agonists. Considering the close association between MDMA use and depression in humans, these results could be significant for human users of ecstasy if the neurotoxicity in FSL rats is reflected in humans.

Finally, rats treated in their home cages with an ambient temperature of 26ºC, instead of the small enclosed area at the end of the thermal gradient showed no increase in T_C after 10 mg/kg MDMA, whereas 10 mg/kg methamphetamine and 5 + 5 mg/kg MDMA + methamphetamine resulted in significant hyperthermia after repeated administrations given twice daily for four days. This is important as it shows how significant the conditions drugs are given in are when looking for hyperthermia. Changes, or lack of changes, in T_C also relate closely to the longer term neurochemical effects which will be discussed below in the section covering neurochemical changes in the brain.

The findings discussed above are the easiest to compare to the existing literature and were mostly expected from looking at previous results. We showed that administration of all stimulant drugs tested increase T_C when given at a high ambient temperature, while MDMA given at temperatures closer to room temperature has no effect on T_C. These results are important as they relate to the thermoregulatory behaviour displayed by rats in the thermal gradient which will be discussed below, and are entirely consistent with human data showing MDMA induced increases and decreases in core temperature (Ling *et al*, 2001; Williamson *et al*, 1997).

Core Temperature at a Non-Constrained Ambient Temperature

The major novel finding of this thesis is the contribution of behavioural thermoregulation to overall control of T_C after administration of MDMA and other stimulant drugs and how this can change under different circumstances. In the first paper we showed that there were not only differences in autonomic thermoregulation in response to a range of stimulant drugs, but also in thermoregulatory behavioural responses when rats were allowed in the runway. We also showed different subsequent T_C during the four hours in the runway in rats treated with the range of drugs used, a phenomenon also shown between the treatment groups in the second and third studies.

In all experiments, rats treated with MDMA chose increasingly cooler areas of the runway with increasing dose for the first 30 minutes, before choosing warmer areas not significantly different to saline treated rats for the remaining time in the thermal gradient. Rats treated with the higher doses, however, appear to behaviourally overcompensate for the drug induced hyperthermia. They exhibit prolonged hypothermia, suggesting they remain in the cold areas of the runway even when their core temperature is below normal. The delay in rats responding to the decrease in temperature in all experiments may be because of a residual feeling of the greatly increased T_C, or possibly a result of the normal range of T_C increasing. These findings still require more investigation to determine the underlying mechanisms. The T_C of MDMA treated SD rats, which had not been given MDMA chronically, does return to normal before the end of the four hour observation period, when preference for the warmer parts of the gradient was shown.

Rats which had been treated with MDMA for 6 weeks before being tested in the thermal gradient also showed the same initial decrease in T_C, but towards the end of the four hours in the runway, T_C had begun to rise above normal again and was significantly higher than that of 1 week rats. This indicates an effect on behavioural thermoregulation by

long term administration of MDMA. No tolerance to the effect of MDMA on hyperthermia at the high ambient temperature was shown, so this was an interesting result which would not have been observed without an investigation on behavioural thermoregulation. There were no differences in T_P in these chronically treated rats during the first two hours in the runway. However, during the time period 120-180 minutes these rats appeared to choose warmer areas on the runway. Choosing a higher temperature on the gradient may contribute to the higher T_C in these rats compared to the rats which were not treated chronically. It also suggests the behavioural response to smaller changes in T_C may be disrupted after taking MDMA for an extended period of time. This could be dangerous as it may lead to a more gradual development of hyperthermia over a few hours which may not be noticed.

FSL rats also showed a similar pattern to SD rats during the first 30 minutes in the thermal gradient after MDMA treatment, with T_C again falling below baseline as soon as they were allowed in the runway, then returning to normal by the end of the four hours. However, when FSL rats were treated with 7.5 mg/kg MDMA, they showed a slower recovery in T_C, despite choosing the cooler areas of the runway, suggesting a possible continued disruption to autonomic thermoregulation. T_C was also lower after 7.5 mg/kg MDMA compared to saline treatment at the end of the four hours in the gradient in FSL rats. Preferred temperature on the runway continued to show no differences between strains or doses until the final two hours in the runway, when FSL rats treated with the high dose of MDMA (7.5 mg/kg) chose the cooler areas on the runway again, which may have been what kept their T_C the same as or lower than other groups during this period. In humans, the pattern of use of MDMA and associated activities, such as dancing, generally extends for a number of hours. Thus, delayed effects of MDMA on thermoregulation, which were reported in both the second and third papers, may be important in understanding adverse effects in humans.

Treatment with other drugs also led to differences in T_C observed during the time in the thermal gradient. As occurred in my honours project (Jaehne *et al*, 2005), PMA treated rats chose warm areas, not significantly different to those chosen by saline treated animals, in contrast to rats treated with any of the other drugs. This behaviour would appear to be inappropriate as their T_C increased greatly in the warm environment and it would be expected that they would prefer a cooler ambient temperature. However, T_C of rats was significantly lower after 26 or 40 µmol/kg (5.25 or 8.08 mg/kg respectively) doses than after saline administration when rats were in the runway. Therefore choosing the warmer areas is probably appropriate to prevent T_C staying low as occurred with the MDMA treated rats.

Methamphetamine treated rats showed the most appropriate behavioural responses to drug induced hyperthermia. The rate of decrease in T_C after rats were allowed in the runway was much slower after the two highest doses, similar to the effect shown in FSL rats after the higher dose of MDMA. If this more gradual change in core temperature occurs in people it could explain why human users have less adverse temperature reactions to methamphetamine as increasing the dose would only result in minor increases in core temperature.

The T_C of cocaine treated rats did not get as high as after other treatments, but the rats still showed a similar behavioural response to increased T_C as after MDMA treatment, with the highest dose leading to the greatest increase in T_C and rats choosing the coolest areas on the runway during the first hour. The T_C of cocaine treated rats also quickly returned to a steady baseline when they were allowed in the runway, and stayed constant for the whole time in the runway.

In view of the fact no other studies have looked at behavioural thermoregulation after MDMA administration, it is difficult to describe how these results fit into the existing literature. Differences which are important to note are firstly, that all studies showed that MDMA treated rats may overcompensate for their high T_C when in the runway, and choose cooler areas for extended periods so that they become hypothermic. Secondly, while rats treated with MDMA, methamphetamine and cocaine show preference for spending more time in cooler areas with increasing dose (and increasing T_C), rats treated with PMA behaved differently and did not spend time in cooler parts of the runway. Thirdly, T_P does not change greatly after chronic treatment, although T_C after four hours in the thermal gradient is higher than rats given MDMA on fewer occasions. Finally, FSL treated rats behave the same as SD rats when first in the thermal gradient, but choose cooler areas after 2-3 hours. We still do not have a clear idea of how behavioural responses contribute to drug-induced hyperthermia except to say that they are not identical for all drugs. The issue of perception of increased core temperature is an obvious question but coming up with an appropriate experimental design for this is difficult and beyond the scope of this thesis.

Cardiovascular and Locomotor Activity Changes

Heart rate (HR) and locomotor activity (LMA) were measured during all experiments as important measures of metabolic contributions to T_C. Most of these data showed results which were expected from looking at the existing relevant literature (Badon *et al*, 2002; Bexis and Docherty, 2006; Dafters, 1994; Green *et al*, 2003). We did however see some interesting results in rats treated chronically with MDMA, as well as in FSL rats. There may also be a relationship between these parameters and the changes in T_C which warrants discussion.

Throughout this thesis we have shown that MDMA administration leads to an increase in HR after a single dose at a high ambient temperature, and no change when

given at normal room temperature. When MDMA was given chronically for 6 weeks, HR during the first week was significantly higher than during subsequent treatment weeks at both ambient temperatures, suggesting tolerance to the effects of MDMA on HR. This was in contrast to the lack of tolerance to the effects of MDMA on T_C and LMA. There has been little work done on the effect of MDMA on HR and other cardiovascular parameters. Badon *et al* (2002) gave SD rats MDMA (3 or 9mg/kg) via i.v. injection twice a day (at 9:00 am & 4:00 pm) for four days on three occasions with 10 MDMA free days in between each. They found that MDMA increased mean arterial pressure (MAP) and produced a biphasic heart rate response. They reported increases in the tachycardic response on the 2nd and 3rd binge compared to the 1st, but no change in the tachycardic response which is in contrast to our HR results after chronic MDMA. I.v. administration of MDMA used by Badon *et al* (2002) would lead to more immediate effects on HR after each administration compared to i.p. administration, which may account for differences between the studies.

We have also shown that there were differences in the MDMA induced HR response of FSL compared to SD rats. The HR of SD rats showed a trend towards increasing with increasing doses for most of the experiment. While baseline HR was the same in both strains, HR increased to a higher level in FSL rats after all doses compared to SD rats. FSL rats also showed significant increases in HR compared to saline after both doses of MDMA for the whole time data were collected. This is consistent with a previous study which showed there were no differences in the diurnal cycle of HR and MAP between FSL and SD rats, but that when rats were given the $5HT_1$ agonist 8-OH-DPAT, HR and MAP of FSL rats rose to a greater level than SD rats (Hildreth *et al*, 2008).

Cardiovascular physiology is important in mammals when they must adapt to changes in ambient temperatures. When core temperature is being maintained at a set point, cardiovascular responses to cold ambient temperatures are closely related to the

mechanism for heat production in rats (Chambers *et al*, 2000), which increases in a parallel fashion with HR. It has been shown in non-human primates that disruption of the preoptic anterior hypothalamus by direct cooling leads to high heart rate and T_C, while warming leads to low HR and T_C (Morishima and Gale, 1972). HR may therefore provide an indirect indication of heat production and disrupted autonomic thermoregulation. This could suggest that the higher HR shown in rats treated at the high ambient temperature in the second paper could be related to the high T_C in these groups. However, HR stays high while T_C falls when these rats are allowed in the runway. Also, rats treated chronically at the high ambient temperature have the same T_C as those treated for only 1 week, while HR is significantly different, which does not fit this hypothesis. In the third paper, an association between HR and disrupted autonomic thermoregulation could suggest that the higher HR shown in FSL rats throughout the time in the thermal gradient indicates that abnormal heat production is occurring. This could then explain why these rats return to cooler areas on the runway during the final 2 hours without any major changes in T_C observed.

It could be also be suggested that the higher HR shown in some rats in the second and third papers may be related to the increased LMA also shown in some groups. In Paper 2 LMA and HR of rats treated at the high ambient temperature was significantly higher than that of rats treated at normal ambient temperature during confinement and the first hour in the thermal gradient as we have reported previously (Jaehne *et al*, 2005). However, like the effect on T_C, the LMA of rats treated with MDMA chronically for 6 weeks is no different to those treated for only 1 week, i.e. no tolerance or sensitisation to MDMA induced increases in LMA is shown. Also like T_C, LMA falls to very low levels, with rats being almost completely inactive, by the end of the time in the runway, while the HR is still very high in the high ambient temperature 1 week group.

A similar relationship was shown in Paper 3 between SD and FSL rats administered MDMA. During confinement to the high ambient temperature both strains showed very low LMA after saline and very high LMA after both doses of MDMA. Higher LMA continued after the high dose of MDMA throughout the time in the thermal gradient in both strains. LMA was also much higher in FSL rats compared to SD rats during confinement to the high ambient temperature, which may have also contributed to the higher T_C and HR in these animals. However, when rats were in the thermal gradient, HR of FSL rats remained much higher than SD rats, while LMA was actually the same or lower than SD rats. The results from both studies suggest that there may be a direct effect of MDMA on HR, as reported by Bexis *et al* (2004), which is independent of T_C and LMA and is heightened in FSL rats.

In summary, the effect of MDMA on T_C appears to be independent of its effects on HR and LMA. While there are times throughout the experiments where these three parameters are all high, some drop when rats are allowed in the runway, while in most other cases, HR stays elevated. This result is consistent in both SD and FSL rats, and is strengthened in the chronically dosed rats which showed tolerance to the HR-increasing effects of MDMA, but not the T_C or LMA-increasing effects.

Neurochemical and Proteomic Changes in the Brain

Researchers have been studying the cellular effects of MDMA for a long time, but it is still not clear what changes precede MDMA-induced neuronal damage (Baumann *et al*, 2007; Cadet *et al*, 2007). Many different markers have been looked at, but none completely explain why depletion of 5HT can occur after chronic use of MDMA. MDMA has previously been shown to result in reduced concentrations of neurotransmitters such as 5HT in the brains of rats after high or repeated doses (Green *et al*, 2003; 2005), or doses

that have induced hyperthermia (Malberg and Seiden, 1998). Some of our studies used repeated dosing and all rats treated at high ambient temperature showed hyperthermia, however we only found limited changes in neurotransmitter concentrations in our studies. This may have been because higher drug doses and different strains were used in some studies, or because hyperthermia was only induced for 30 minutes at any time so may not be long enough to elicit major changes in brain concentrations of neurotransmitters affected by MDMA and other stimulant drugs.

The treatment regimes used to investigate the dose-response relationship of MDMA, PMA, methamphetamine and cocaine with thermoregulation, showed no statistically significant changes in cortical concentration of 5HT, 5HIAA, DA or DOPAC from untreated controls in any of the four drug treatment groups, although a non significant reduction in 5HT was noted after MDMA administration. The repeated dosing regimen we used in Paper 2 also led to no statistically significant changes in cortical concentrations of 5HT or DA in any of the treatment groups. However, both groups of rats that were administered MDMA for 6 weeks showed significant decreases in 5HIAA and DOPAC, the major metabolites of 5HT and DA, compared to controls. Decreased concentrations of 5HIAA and DOPAC in the brain may suggest that there is less turnover of 5HT and DA occurring. It should also be noted that decreases in 5HIAA levels following MDMA administration without changes in 5-HT levels has been reported by other workers (Malberg and Seiden, 1998).

We did, however, see changes in neurotransmitter concentrations of FSL rats treated with MDMA at a high ambient temperature. Baseline tissue concentrations of 5HT, 5HIAA, DA and DOPAC in the cortex were similar in untreated SD rats compared to untreated FSL rats. One week after the treatment and dosing regimen used in this study, cortical tissue concentration of neurotransmitters measured did not change in SD rats. In contrast, FSL rats treated with MDMA showed decreases in all neurotransmitters

measured. This could suggest that FSL rats are more susceptible to the neurotoxic effects of MDMA. However, as only 3 FSL rats survived all the treatments in this study to be used in this analysis, it is difficult to be confident about this observation. More rats could not be used due to ethical concerns about the lethality rate of the treatment used. The result could also reflect the decreases reported after chronic anti-depressant treatment reported in other papers (Zangen *et al*, 1997; 1999). However, these decreases were normalisations of previously elevated concentrations, and likely represent a different drug effect.

Proteomics appeared the ideal method to look at the changes in protein expression which may precede greater damage in the brain, and be useful in formulating new hypotheses about the early cellular effects of MDMA, which may precede and lead to more overt neurotoxicity as measured by loss of neurotransmitters or other neuronal markers. We therefore compared the usual measures of MDMA effects, T_C and changes in neurotransmitters, to a proteomic profile of the rats' brains. This study used a very different dosing regimen and protocol than the first three papers, designed to induce early low level neurotoxicity. This was because we wanted to identify early changes in protein expression leading to terminal degeneration, rather than merely confirm that major terminal damage had occurred. Methamphetamine was used to contrast dopaminergic damage with MDMA-induced 5HT damage and provide some indication of neuronal specificity. Rats treated with the combination MDMA + methamphetamine treatment showed significant decreases in cortical concentrations of 5HT and 5HIAA, and also had significantly lower striatal concentrations of 5HT than methamphetamine treated rats two weeks after drug treatment. This is in contrast to MDMA treated rats, which showed no decreases in 5HT or 5HIAA in either brain region measured. This was expected as other papers have shown hyperthermia is required to see significant changes in neurotransmitter concentration after single doses (Malberg *et al*, 1996; Malberg and Seiden, 1998), although

lack of hyperthermia was not expected. We also showed no significant changes in 5HT or 5HIAA concentrations in the brains of methamphetamine treated rats, which was also expected as previous studies suggest that more frequent dosing is required to see long term changes in these neurotransmitters (Bowyer *et al*, 1994; Broening *et al*, 2005; Clemens *et al*, 2007; 2004). A trend towards a decrease in DA was shown in the cortex of rats treated with methamphetamine or MDMA + methamphetamine.

Some unexpected results were seen when the proteomic analysis of the cortex was performed. While the thermoregulatory and neurochemical effects were greatest compared to saline in the MDMA + methamphetamine group, most of the protein changes were actually shown in MDMA or methamphetamine treated rats, suggesting there was no clear relationship between the neurochemical and proteomic results. However, several identified proteins were of interest because of their function and roles in disease. These included aconitate hydratase, mitochondrial (ACON), which is involved in breakdown of citrate, up-regulated in the methamphetamine group, ubiquitin-conjugating enzyme E2 variant 1 (UB2V1), a novel regulator of protein ubiquitination, down-regulated in the MDMA group, and dual specificity mitogen-activated protein kinase 1 (MEK1), which is involved in signal transduction, as well as other important proteins, which showed changes in both the MDMA and methamphetamine groups. Also showing changes were glutathione transferase omega-1 (GSTO1), which showed the biggest changes in expression with an increase after MDMA treatment. There were however differences between the MDMA + methamphetamine group and the other two drug treatment groups including ATP synthase B chain, mitochondrial precursor, which is involved in cell respiration, as well as the proteins mentioned above MEK1, ACON, and UB2V1. All of the changes in protein abundance were modest but this should not be taken as biologically unimportant. It is reasonable to expect that early drug induced changes in synaptic proteins may be subtle

with gross changes in protein expression only occurring when significant pathology is evolving.

In summary, we have shown that administration of MDMA and other stimulants to rats in our experimental protocol of behavioural thermoregulation has little effect on cortical concentrations of 5HT or DA, except in FSL rats. As will be discussed in the next section, our results should be extended to look at other markers of damage in the brain caused by these drugs under different circumstances. Our study of proteomics was an attempt to start doing this, although only limited proteins were found which showed changes after the treatment regiment used. Identifying early effects of MDMA on neuronal protein expression may require further development of proteomic techniques to improve sensitivity.

Limitations and Future Directions

As with many studies, there are limitations to the work that has been conducted in this thesis which should be addressed in the future. In the third paper we conducted a simple experiment to compare the blood and cortex concentrations of MDMA and its metabolite MDA between SD and FSL rats under normal housing conditions (room temperature, home cage) over time. This showed that there were no differences between strains in the metabolism of MDMA and that concentrations achieved in our experiments are comparable to those seen in human users (Irvine *et al*, 2006). It would be important to repeat this type of experiment after different doses of MDMA, and given under the high ambient temperature conditions used in most of our experiments, as well as in rats given access to the thermal gradient. This would let us see how changes in T_C affect the metabolism of the drug, and how this in turn could affect thermoregulation in the animal. It has been shown that the metabolism of other drugs can differ depending on T_C. For

example, Yuan *et al* (2006) showed that, after administration of methamphetamine at a high ambient temperature, Squirrel monkeys that had the highest increase in T_C, also had the highest plasma concentration of the metabolite amphetamine. However, plasma methamphetamine concentration did not change depending on T_C.

To determine possible mechanisms for the effects we have reported throughout this thesis, future experiments should incorporate co-administration of drugs which may prevent the effects of MDMA. This would include anything that acts on the 5HT or DA systems, such as receptor agonists and antagonists, drugs that block reuptake of neurotransmitters such as selective serotonin reuptake inhibitors (SSRI) and dopamine transporter (DAT) blockers, and drugs that prevent the breakdown of neurotransmitters such as monoamine oxidase inhibitors. Previous studies with these types of drugs have shown that a DA D_1 receptor antagonist prevents MDMA induced hyperthermia (Mechan *et al*, 2002), as does a $5HT_{2A}$ antagonist and a tyrosine hydroxylase inhibitor (Malberg *et al*, 1996) and clozapine, an atypical antipsychotic (Blessing and Seaman, 2003).

Other options for furthering the results include looking at the drug administration schedules used throughout the thesis, as well as the conditions under which drugs were given in each experiment. For example, in the repeated dosing experiment, we attempted to simulate a type of weekend dosing regimen, over three consecutive days each week. Other researchers also try to simulate another type of human use pattern by giving multiple doses on the same day (Green *et al*, 2004a). The problem with these dosing regimens is that they may be too simplistic, as rats have much shorter life spans than humans. Rats also have different metabolism, so repeated doses in the same day is also unlikely to directly simulate repeated dosing in humans (de la Torre and Farre, 2004). With a better understanding of pharmacokinetic factors, more appropriate dosing regimens, closely related to human use, can be used.

The most important aspect that should be expanded on in the future, which we only looked at in a limited way in this thesis, is the long term effects of MDMA and other stimulants on the brain in relation to thermoregulation. Although we showed very few changes in cortical concentrations of 5HT, DA or their metabolites throughout these experiments, perhaps if other brain regions more closely associated with thermoregulatory function were looked at, such as the preoptic anterior hypothalamus (POAH), it would have revealed drug effects on the concentration of these neurotransmitters. In the experiments where changes were shown, i.e. decreased 5HIAA and DOPAC after chronic dosing, and decreases in all neurotransmitters in FSL rats, other markers of neurotoxicity should be measured, including 5HT transporters, receptors and neurons. Proteomic measurements could also be extended to different brain regions, and also be investigated after different doses, dosing regimens and conditions, as well as over different time courses from final drug administration.

Conclusions

In response to the first aim of this thesis "to investigate the acute effects of MDMA and other stimulant drugs on behavioural thermoregulation and related physiological parameters", we firstly established dose response relationships for increases in T_C induced by MDMA and other stimulant drugs in a warm ambient temperature and showed there are quantitative and qualitative differences in how these drugs influence thermoregulation which appears to be in accordance with human case reports. We subsequently showed that long term treatment with MDMA results in an apparent tolerance to the effects of the drug on HR, while showing no change in its effect on T_C and LMA when administered at a set ambient temperature. We have also shown that MDMA treatment at a high ambient temperature leads to a higher T_C and more fatalities due to hyperthermia in FSL compared

to SD rats, as well as greater MDMA-induced increases in LMA and HR at a high ambient temperature.

In addition, we have also shown differences in the behavioural response of rats when comparing MDMA administration to other stimulant drugs and between various dosing regimens and conditions. Appropriate thermoregulatory behavioural responses to initial large changes in body temperature were shown after cocaine and methamphetamine administration but MDMA and PMA administration led to different behaviour by the rats. Studying the effect of repeated administration of MDMA showed that there is some disregulation in overall ability of rats to control their T_C properly after long term exposure to MDMA. We also showed important differences in behavioural thermoregulation between SD and FSL rats.

In response to the second aim of this thesis "to investigate the residual neurochemical changes caused by MDMA and other stimulants", few changes were reported throughout these studies in cortical concentration of neurotransmitters affected by MDMA and other stimulant drugs. The dosing regimens used in all studies led to no significant changes in 5HT or DA after MDMA or any other drug given alone, at any ambient temperature in SD rats. Decreased concentration of 5HIAA and DOPAC was shown after 6 weeks of repeated MDMA dosing at both high and normal ambient temperature, and decreases in concentration of all neurotransmitter measured were reported in MDMA treated FSL rats. MDMA + METH given in combination twice a day for four days also led to significant decreases in 5HT and 5HIAA in the cortex of SD rats, although these results did not correlate well with proteomic changes shown in this study.

Overall, the results of this thesis suggest that animal models which incorporate both physiological and behavioural measures of thermoregulation may be more appropriate in

investigating the pharmacodynamics and mechanisms underlying stimulant induced hyperthermia. This model may also allow better translation and comparison with human data. The results of the repeated dosing study may be important in users of 'ecstasy' when considering the cardiovascular risks of taking the drug and possible tolerance to the rewarding effect of the drug. We have also shown for the first time in a rat model of depression, that these animals show a higher susceptibility to developing MDMA-induced hyperthermia and depletion of neurotransmitters in the brain. Considering the close association between MDMA use and depression in humans, and the difficulty in assigning cause and effects, further investigations into the relationship between depression and MDMA-induced drug effects are warranted.

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