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NEUROPHARMACOLOGICAL PROPERTIES OF THE CATHINONES

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Abstract

At the height of its popularity, mephedrone was the most common recreationally used cathinone. This is thought to be due to its perceived likeness to MDMA. Therefore the aim of this thesis was to examine mephedrone-induced changes in behaviour, body temperature or neurochemistry in the rat and to compare these changes to those observed following MDMA administration. This was achieved by assessing changes in body temperature following acute mephedrone, MDMA, cathinone or methcathinone administration. Additionally, locomotor activity and cognitive tasks were performed following chronic intermittent administration of mephedrone, MDMA or cathinone. These behaviours, as well as mephedrone-induced changes to body temperature and 'anxiety-related' behaviour, were also assessed following either pre-treatment with MDMA or co-administration of caffeine. Finally, locomotor activity, body temperature changes and *in vivo* striatal dopamine release were assessed following rapid repeated dosing of mephedrone, and the roles of dopamine, 5-HT and noradrenaline in these responses were examined. Post mortem monoamine concentrations from specific brain regions were also assessed following acute, chronic intermittent and rapid repeated dosing of mephedrone.

It was found that the neurochemical, behavioural and physiological effects of mephedrone in the rat include hyperactivity, hypothermia, cognitive deficits, anxiety-related behaviour and increased striatal dopamine efflux. Pre-exposure to MDMA, or concomitant caffeine administration, caused an increase in rectal temperature following mephedrone injection while caffeine co-administration prolonged the hyperactive profile of mephedrone. Importantly, unlike MDMA, rapid repeated mephedrone administration (3 x 10 mg kg^{-1} at 2 h intervals) had no cumulative effect on mephedrone-induced hypothermia or hyperactivity. It is also clear that mephedrone is inducing its effects via noradrenergic, dopaminergic and serotonergic mechanisms. The cathinones and MDMA had varying effects on post mortem tissue levels of the monoamines and their metabolites. Importantly, these effects of mephedrone appear to be occurring by mechanisms that are different, but similar, to MDMA.

Publications

Papers

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Shortall SE, Worth R, Garner P, Fone KC, Green AR, King MV. Pre-exposure to MDMA during early adolescence has differential effects on the behavioural, neurochemical and temperature responses to mephedrone in the rat. *In preparation.*

Shortall SE, Green AR, Swift KM, Fone KC, King MV (2013). Differential effects of cathinone compounds and MDMA on body temperature in the rat, and pharmacological characterization of mephedrone-induced hypothermia. *British Journal of Pharmacology* 168(4): 966-77.

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Abbreviations

5-HIAA – 5-hydroxyindoleacetic acid

5-HT – 5-hydroxytrptamine

5,7-DHT – 5,7-dihydroxytrptamine

6-OHDA – 6-hydroxydopamine

ANOVA – Analysis of variance

Cath – Cathinone

CER – Conditioned Emotional Response

DAT – Dopamine transporter

DOPAC – Dihydroxyphenylacetic acid

FCtx – Frontal cortex

Hip – Hippocampus

HPLC-ED – High performance liquid chromatography with electrochemical detection

HVA – Homovanillic acid

Hyp – Hypothalamus

i.c.v. – intracerebral ventricular

i.p. – Intraperitoneal

ITI – Inter-trial interval

LMA – Locomotor activity

NET – Noradrenaline transporter

NOD – Novel object discrimination

MAO – Monoamine oxidase

MDMA – 3,4-methylenedioxymethamphetamine

Meph – Mephedrone

Methcath – Methcathinone

mg kg^{-1} – Milligrams per kilogram of body weight

ml kg^{-1} – Millilitres per kilogram of body weight

PCA – Perchloric acid

PPI – Prepulse inhibition of the acoustic startle response

s.c. – Subcutaneous

SEM – Standard error of the mean

SERT – 5-HT transporter

Str – Striatum

TH – Tyrosine hydroxylase

TPH – Tryptophan hydroxylase

VMAT – Vesicular monoamine transporter

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Chapter 1 General Introduction

Cathinone is a naturally occurring psychostimulant found in the leaves of the Khat plant. Recent years have seen the development of synthetic derivatives of this compound known as 'designer cathinones' or simply 'cathinones'. The term 'designer drug' is used to describe psychostimulants or other drugs of abuse that are used illicitly and produced from the structures of existing drugs. The designer cathinones have structures similar to the amphetamines and are taken recreationally for their psychostimulant effects. The cathinone derivative mephedrone is a popular recreational drug which has received a lot of media attention, but little was known on its pharmacology or adverse effects at the commencement of the studies presented in this thesis. The aim of this thesis is to perform a preclinical examination of the effects of selected synthetic cathinones in rats, with a particular focus on mephedrone, and to compare these effects to 3,4-methylenedioxymethamphetamine (MDMA).

MDMA is a ring substituted amphetamine with a methylenedioxy substitution at positions 3 and 4 of the aromatic ring of the amphetamine molecule (Fig 1.1). The cathinones are structurally similar to amphetamine with the only difference being a substituted ketone group at the β position on the side chain (Fig 1.1). Cathinone and methcathinone are β-keto derivatives of amphetamine and methamphetamine, respectively (Fig 1.1). Mephedrone, however, does not have a commonly used amphetamine analogue.

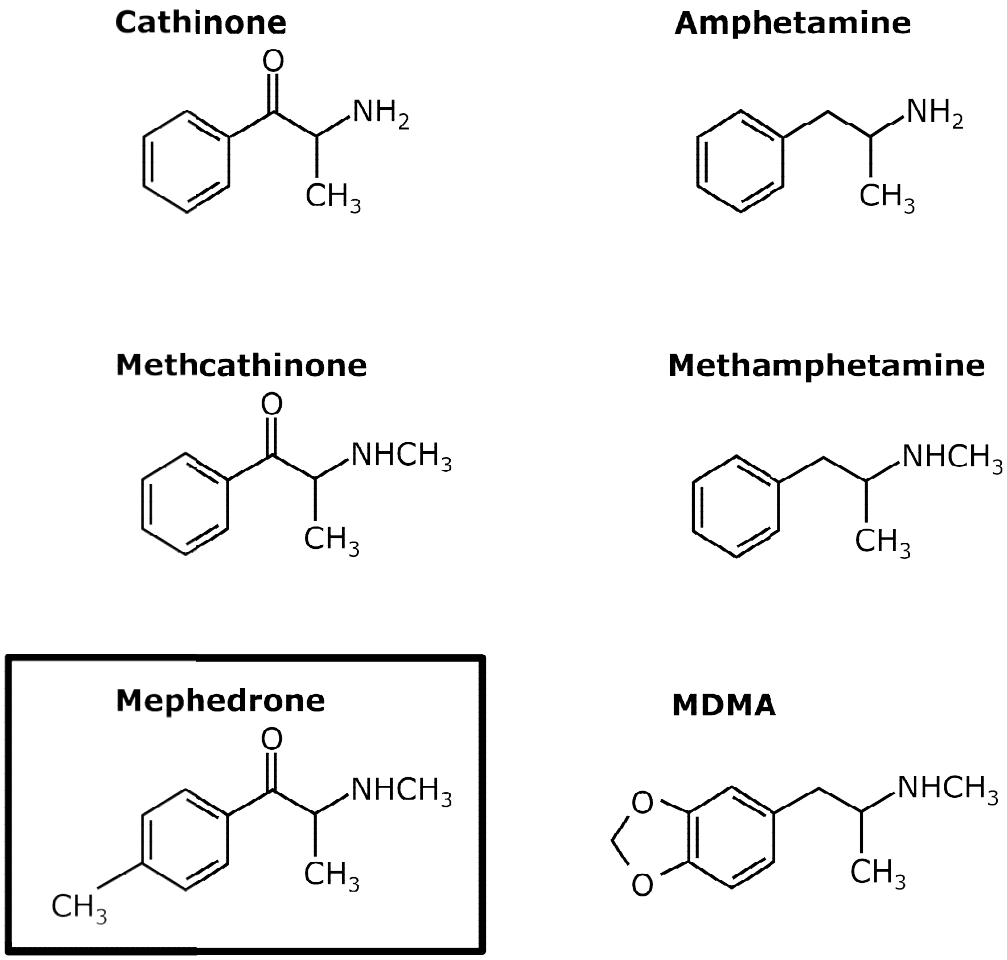


Figure 1.1 Chemical structures of the cathinones examined in this thesis and related amphetamines.

1.1 History and legality of substituted amphetamines

MDMA first became popular as a recreational drug amongst the dance-club scene in the 1980s (Green et al. 2003), however, it was first synthesised by Merck in 1912 and patented as an intermediary for the synthesis of therapeutically active compounds (Freudenmann et al. 2006). Due to its empathogenic effects, MDMA was considered as a pharmacological aid in psychotherapy in the 1950s, and is currently being investigated as a treatment for post traumatic stress disorder (Green et al. 2003; Mithoefer et al. 2013; Oehen et al. 2013). The Misuse of Drugs Act, 1971 was established in the UK to prevent the non-medical use of harmful drugs and to classify these drugs in accordance to their potential for causing harm to

both the individual and society as a whole. Most amphetamines are classified as Class B drugs under this act however MDMA was declared a Class A drug in 1977 due to its abuse potential (ACMD 2008). In recent years the purity of ecstasy tablets has declined due to strict restrictions on its manufacturing and distribution. For example, less than 50% of ecstasy tablets confiscated in the Netherlands in 2009 contained MDMA as their primary component, compared to 90% in previous years and, in the US, results from an online testing service revealed that 39% of ecstasy tablets contained MDMA only from 1999-2005 (Tanner-Smith 2006; Brunt et al. 2011). It is thought that this decline in purity and availability is one factor that has led to the increased popularity of MDMA-like 'legal highs' in recent years.

Unlike MDMA, cathinone is a naturally occurring compound. The leaves of the Khat shrub (*Catha eludis*), originating from the Arabian peninsula and East Africa (ACMD 2010), are commonly chewed or sometimes brewed as tea for their psychostimulant effects. Traditionally khat use was confined to areas surrounding its natural habitat (for example Sudan, Ethiopia, Yemen and Somalia) as it must be used fresh to obtain its desired effects (Feyissa and Kelly 2008). However, improved transportation, as well as the movement of ethnic groups outside of the plant's natural habitat, has led to its more recent introduction to the United States, Canada, Australia and Europe in the form of harvested leaves (Feyissa and Kelly 2008). Cathine was isolated from khat leaves in 1930 and was initially thought to be the main active psychostimulant component in these leaves. However, since it had only modest stimulant properties further investigation was conducted, leading to the discovery of the much more potent stimulant, cathinone, in 1975. The World Health Organisation labelled the active constituents of khat, namely cathinone and cathine, as schedule I and schedule III substances, respectively, in 1971 (Feyissa and Kelly 2008). The khat plant remains legal in many African countries, but is illegal in other countries such as France, Germany, Ireland, Canada and the US, and very recently in the UK, amongst others. Cathinone is now a class C drug in the UK and both methcathinone and mephedrone are classified as class B drugs although at the time of the ban very little scientific evidence existed to indicate that either is more harmful to the individual than cathinone.

The synthetic cathinone derivatives have recently received substantial media attention, but they are not newly discovered compounds. Methcathinone was first synthesised in the late 1920s and originally marketed as an antidepressant in the Soviet Union in the 1930s and 40s, and subsequently developed as a potential appetite suppressant but never marketed as such due to its addictive potential (Kelly 2011). Methcathinone was declared illegal in several countries in the 1990s following its widespread abuse. Additionally, although considered a designer drug, mephedrone was first synthesised in 1929 but it received little interest until the late 2000s, when it started being sold on the internet as 'plant food' and 'bath salts' and in high street head shops as a 'legal high'. This coincided with a decrease in the availability of MDMA and also in the purity of 'ecstasy' tablets (Tanner-Smith 2006; Brunt et al. 2011). In many of these tablets MDMA was substituted by other compounds and in 2009 mephedrone was found to be one of the most prevalent new designer drugs to be misleadingly sold as ecstasy (Brunt et al. 2011).

Although mephedrone has been implicated in a number of deaths and became illegal in Europe and the USA between 2010 and 2012 (Dargan et al. 2011; Gershman and Fass 2012) it remains available and popular for illicit use (McElrath and O'Neill 2011; Ayres and Bond 2012; Van Hout and Bingham 2012; Kelly et al. 2013; Yamamoto et al. 2013). Before being classified as an illegal substance, mephedrone was the most popular of the cathinone derivatives used recreationally because of its reported psychostimulant properties and empathetic effects. One online survey of 2289 experienced polydrug users conducted in 2009 showed 42% had tried mephedrone at least once, with approximately 30% using it every two weeks or more frequently (Winstock et al. 2011). A recent review of new psychoactive drugs detected in post-mortem and criminal casework collated between 2010 and 2012 in the UK demonstrated that of 203 cases mephedrone was identified in 106 cases (Elliott and Evans, 2014; Fig 1.2). Recreational users of mephedrone have compared its stimulant effects to those of MDMA and cocaine (Winstock et al. 2011) and some users considered mephedrone to be superior to MDMA (Vardakou et al. 2011).

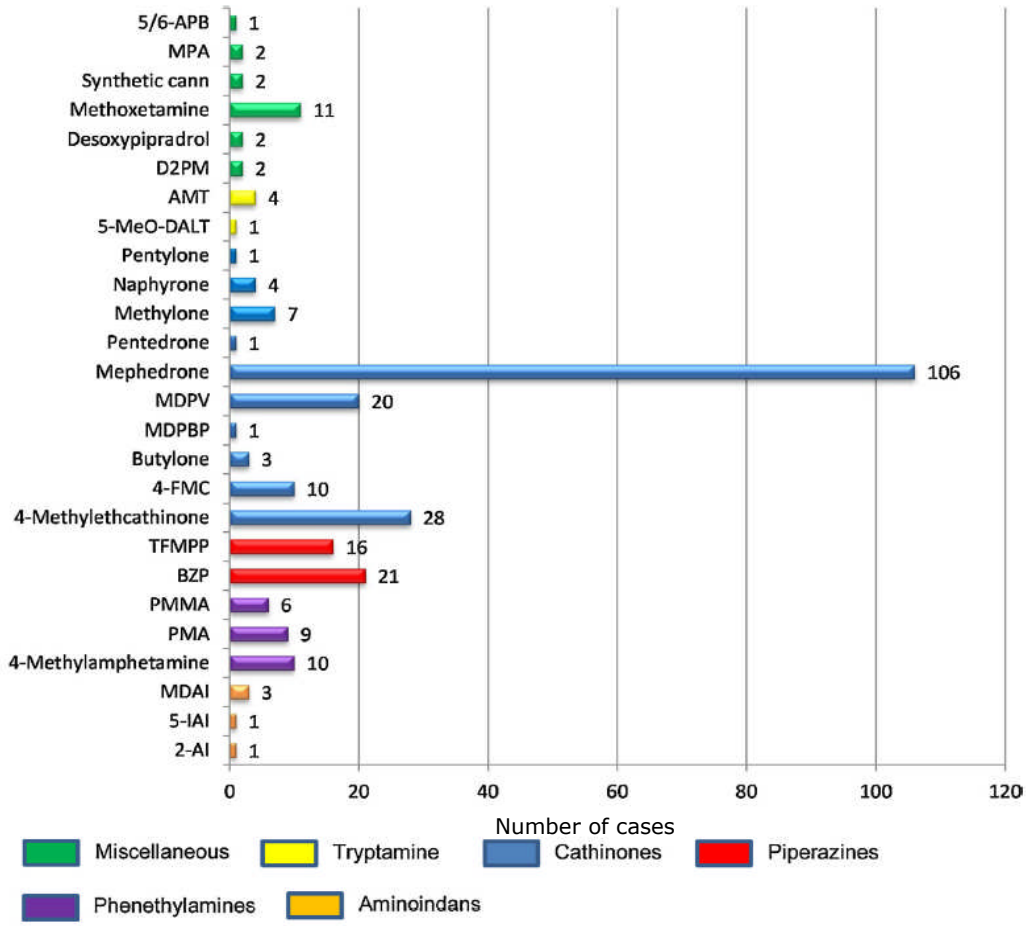


Figure 1.2 Mephedrone was the most popular new psychoactive substance identified in post-mortem and criminal casework between 2010 and 2012 in the UK (n=203 cases).

Figure taken from (Elliott and Evans 2014).

1.2 Metabolism and pharmacokinetics

MDMA is most commonly consumed orally in the form of ecstasy tablets, which typically contain between 80 and 150 mg of MDMA but as previously stated the composition and purity of these tablets can vary (section 1.1). An oral dose of 1 mg kg^{-1} to MDMA users produces a peak plasma concentration (C_{max}) of 150 ng ml^{-1} 2 h post-administration, with a half-life of 5.5 h, and a clearance of 620 ml h^{-1} kg^{-1} (Desrosiers et al. 2013). Similar data have been found in other studies (Mas et al. 1999; Kolbrich et al. 2008). In rats, an oral dose of 2 mg kg^{-1} gives a C_{max} of 46 ng ml^{-1} at 34 min post-administration, with a much shorter half-life of 46 min (Baumann et al. 2009). MDMA mainly undergoes both phase I and phase II metabolism in the liver by O-demethylenation to form 3,4-dihydroxymethamphetamine (HHMA) by the CYP450 enzyme, CYP2D6 in humans. HHMA is then O-demethylated to 4-hydroxy-3-methoxymethamphetamine by catechol-O-methyltransferase. MDMA is also N-demethylated to form 3,4-methylenedioxyamphetamine (MDA), which is subsequently metabolised to 3,4-hydroxyamphetamine and then to 4-hydroxy-3-methoxyamphetamine (Rietjens et al. 2012). In rats, at low doses the formation of MDA is one of the main metabolic pathways, where as in humans the formation of HHMA is predominant (de la Torre and Farre 2004; Baumann et al. 2009). Not only is MDMA metabolised by CYP2D6 but it is also inhibited by it, resulting in non-linear kinetics with inhibition occurring within 1 h (Yang et al. 2006). In humans, there is an increased gradient in the dose-plasma drug concentration slope for MDMA such that a two-fold increase in dose gives rise to a four-fold increase in plasma concentration (Green et al. 2012). In contrast, there is a linear relationship between dose and plasma concentration in rats, which is lost at higher doses, so that the dose-plasma concentration curves in humans and rats are not very different at low doses but vary markedly at higher doses. Administration of a second dose of MDMA 24 h later to humans causes an increase in plasma concentration disproportionate to that observed following the first, due to accumulation of the drug and auto-inhibition of its own metabolism, which may account for the increased cardiovascular effects and acute toxicity observed with repeated dosing (Farre et al. 2004).

As previously stated, the most common route of administration of cathinone is by consuming the leaves of the plant, typically by chewing the fresh leaves, but sometimes the dried leaves are brewed as tea or very rarely smoked. Since the leaves contain low concentrations of cathinone and cathine, large quantities (100-500 g) must be chewed to obtain its desired stimulant effects, meaning that sessions can last for several hours. In healthy, non-drug using volunteers, the peak plasma concentration of cathinone (C_{max}=58.9 ng ml^{-1}) after chewing 36-59 g of khat leaves for 1 h (0.63 mg kg^{-1} of cathinone) was reached 2.3 h after the start of chewing and the terminal elimination half-life was 1.5 h (Toennes et al. 2003). A separate study where volunteers chewed 54-71 g of khat leaves for 1 h (0.8 mg kg^{-1} of cathinone) found the peak plasma concentration of cathinone to be 127 ng ml^{-1} at a T_{max} of 2.1 h, with a terminal elimination half-life of 4.3 h (Widler et al. 1994). The same group found similar effects of a pure cathinone dose of 0.5 mg kg^{-1} administered in gelatine capsules, the only exception being a more rapid T_{max} following administration of pure cathinone presumably due to delayed absorption of cathinone by chewing the leaves (Brenneisen et al. 1990).

Methcathinone is typically taken by the intranasal or oral route and users tend to re-dose several times a day, taking between 1 and 3 g per day for a number of consecutive days (McCann et al. 1998). There is no information on the pharmacokinetics of methcathinone in either humans or laboratory animals.

Like methcathinone, mephedrone is consumed orally or intranasally with a typical oral dose being in the region of 100-200 mg. This oral dose is similar to a typical MDMA oral dose of 1-2 tablets (140-180 mg), however, due to the short duration of action, mephedrone users tend to rapidly re-dose meaning that they can consume 1 g or more of mephedrone in a single session which most typically last for 3-12 h (Winstock et al. 2011). Plasma concentrations of mephedrone in users following fatal doses are approximately 2000 ng ml^{-1}, which is very similar to concentrations following fatal MDMA toxicity (Maskell et al. 2011; Schifano et al. 2012). In Sprague-Dawley rats, a 5.6 mg kg^{-1} subcutaneous (s.c.) dose of mephedrone gave a peak plasma concentration of 1206 ng ml^{-1} with a t_{max}

of 0.25 h (Miller et al. 2013). In rapid repeated dosing studies where Sprague-Dawley rats received a total of four injections of mephedrone at a dose of either s.c. 10 or 25 mg kg^{-1} at 2 h intervals, plasma levels were found to be 384.2 and 1294 ng ml^{-1} 1 h after the final injection (Hadlock et al. 2011). In the same study, whole brain tissue levels of 2.1 ng mg^{-1} and 7.8 ng mg^{-1} were found 1 h after the final injection. Mephedrone has greater blood-brain permeability than MDMA (Simmler et al. 2013). Peak brain levels of mephedrone (4 ng mg^{-1}) were observed 2 min after a 1 mg kg^{-1} i.v. injection and rapidly reduced to 0.4 ng mg^{-1} by 60 min post-injection (Aarde et al. 2013).

Cathinone and methcathinone are metabolised to norephedrine and norpseudoephedrine by Phase I reduction of the β-keto moiety to an alcohol (Brenneisen et al. 1990; Widler et al. 1994; Kelly 2011). Like MDMA, mephedrone undergoes hepatic metabolism and CYP2D6 is the main enzyme involved (Aarde et al. 2013; Pedersen et al. 2013). Mephedrone is metabolised to nor-mephedrone, nor-dihydro-mephedrone, hydroxytolyl-mephedrone and nor-hydroxytolyl-mephedrone by N-demethylation to the primary amine, reduction of the keto moiety to the respective alcohol and, oxidation of the tolyl moiety to the corresponding alcohols (Meyer et al. 2010). These metabolites were identified in Wistar rats after oral dosing, but an additional metabolite, 4-carboxy-dihydro mephedrone was identified in human urine following an oral dose. It is unclear whether any of these metabolites are pharmacologically active.

1.3 Neurochemistry

1.3.1 Monoamine transporters and receptors

MDMA binds to the 5-hydroxytrptamine (5-HT; SERT), dopamine (DAT) and noradrenaline (NET) transporters, having the highest affinity for NET SERT in mice and rats (Battaglia et al. 1988). While MDMA-induced 5-HT release occurs by binding and reversal of the 5-HT transporter, it is thought that MDMA releases dopamine by entering the dopamine terminal by diffusion since the dopamine uptake inhibitor, GBR 12909, does not

block dopamine release following MDMA administration to mice (O'Shea et al. 2001; Camarero et al. 2002). MDMA is also a substrate for the vesicular monoamine transporter (VMAT) so once in the cell it may enter the vesicles via VMAT and deplete vesicular storage by reversal of VMAT activity (Capela et al., 2009, see Fig 1.3 for schematic diagram). Equally MDMA increases extracellular levels of the monoamines by inhibiting the monoamine metabolising enzyme, monoamine oxidase (MAO). MDMA also has a high affinity (K_i<10 μM) for α_2-adrenoceptors, 5-HT_2, M_1 muscarinic and H_1 histamine receptors (Table 1.1), with less affinity (K_i<10-100 μM) for α_1- and β- adrenoceptors, 5-HT_1, M_2 muscarinic, dopamine D_2 receptors and a low affinity for dopamine D_1, μ, δ and κ opioid and benzodiazepine receptors (Battaglia et al. 1988). More recently it has also been found that MDMA has an affinity for the rat trace amine 1 receptor as well as for central nicotinic acetylcholine receptors, particularly the α7 nicotinic acetylcholine receptor (K_i=0.7 μM) (Bunzow et al. 2001; Garcia-Rates et al. 2010).

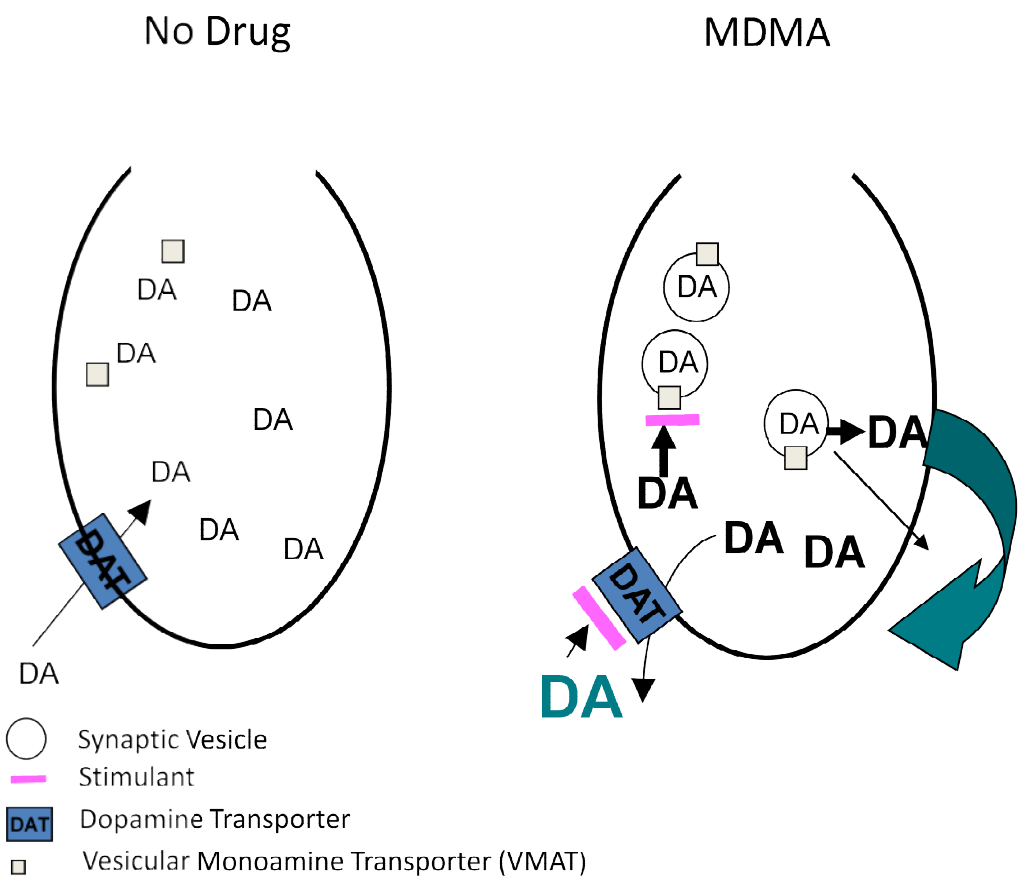


Figure 1.3 MDMA causes dopamine release via reversal of the vesicular monoamine transporter.

MDMA enters the pre-synaptic vesicles via the vesicular monoamine transporter (VMAT) to deplete vesicular neurotransmitter storage by reversal of transporter activity (Capela et al. 2009). DA=dopamine. Modified from Easton et al. (2007).

Table 1.1 Human transporter and receptor binding affinities of the cathinones examined in this thesis and MDMA.

	MDMA	Mephedrone	Cathinone	Methcathinone
SERT	0.61 ± 0.05[1]	>30[2]	>30[2]	>30[2]
DAT	15.8 ± 1.7[1]	3.4 ± 0.8[2]	19.8 ± 1.9[2]	1.28 ± 0.2[2]
NET	24.4 ± 1.9[1]	>25[2]	3.5 ± 2.7[2]	1.45 ± 0.7[2]
5-HT$_{1A}$	12.2 ± 0.8[2]	>20[2]	>20[2]	12.7 ± 3.5[2]
5-HT$_{2A}$	7.8 ± 2.4[2]	2.1 ± 0.7[2]	>13[2]	3.0 ± 0.6[2]
α_{1A}	18.4 ± 1.2[1]	3.48 ± 2.2[2]	5.4 ± 1.1[2]	3.93 ± 1.3[2]
α_{2A}	3.6 ± 0.8[1]	11.0 ± 5.0[2]	8.9 ± 2.7[2]	11.9 ± 3.9[2]
β	19.2 ± 2.1[1]	*NA*	*NA*	*NA*
D$_1$	148 ± 14[1]	>13.6[2]	>13.6[2]	>13.6[2]
D$_2$	95 ± 15[1]	>30[2]	>30[2]	>30[2]
H$_1$	5.7 ± 2.4[1]	>14.4[2]	>14.4[2]	>14.4[2]
TA$_{1Rat}$	0.37 ± 0.12[2]	4.3 ± 2.0[2]	2.2 ± 0.7[2]	4.1 ± 1.2[2]
M$_1$	5.8 ± 0.3[1]	*NA*	*NA*	*NA*
M$_2$	15.1 ± 0.1[1]	*NA*	*NA*	*NA*

Values are K_i (μM); *NA,* data not available; [1]Battaglia et al., (1988), [2]Simmler et al., (2013)

Cathinone and methcathinone have low micromolar range (<10 μM) affinity for DAT and NET, which is similar to amphetamine and methamphetamine (Simmler et al. 2013). Mephedrone, like MDMA, also causes non-selective release of monoamines by acting as a substrate for SERT, DAT and NET, however it is unlikely that it causes neurotransmitter release via VMAT as its effects on this transporter occur at high micromolar concentrations that are unlikely to occur *in vivo* (Hadlock et al. 2011; Baumann et al. 2012; Lopez-Arnau et al. 2012; Martinez-Clemente et al. 2012; Eshleman et al. 2013; Simmler et al. 2013; Opacka-Juffry et al. 2014). Mephedrone has a high affinity (K_i<10 μM) for α_{1A}-adrenoceptors, 5-HT_{2A} and rat trace amine 1 receptors (Table 1.1), with lower affinity (K_i<10-100 μM) for α_{2A}-adrenoceptors (Simmler et al. 2013). While cathinone and methcathinone show similar affinities for α_{1A}- and α_{2A}-adrenoceptors and the rat trace amine 1 receptor, methcathinone also binds to the 5-HT_{1A} and 5-HT_{2A} receptors.

1.3.2 Brain monoamines

5-HT modulates many central and peripheral processes including regulation of sleep, appetite, mood, sensory transmission, gastrointestinal function and blood flow. In the rat, MDMA causes rapid release of 5-HT in a dose dependent manner which leads to reduced tissue concentrations of both 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the first few hours following administration (Stone et al. 1986; Battaglia et al. 1988; Crespi et al. 1997; McCann et al. 2000; Ricaurte et al. 2000; Mechan et al. 2002; Green et al. 2003; Hall and Henry 2006). 5-HT release has been demonstrated following MDMA administration to rat brain synaptosomes and slices (Berger et al. 1992; Crespi et al. 1997) and *in vivo* microdialysis studies have identified a rapid 5-HT release in the striatum, frontal cortex and hippocampus (Yamamoto et al. 1995; Gudelsky and Nash 1996; Mechan et al. 2002). An acute dose of MDMA also inhibits the activity of the rate limiting enzyme for 5-HT production, tryptophan hydroxylase (TPH) in rat striatum, hippocampus, hypothalamus and frontal cortex, as well as the metabolising enzyme, MAO (Stone et al. 1986; Schmidt and Taylor 1987; Stone et al. 1987; Che et al. 1995; Hall and Henry 2006). In mice, however, there is a small decrease in tissue hippocampal and cortical 5-HT 3 h after 3 x 20 or 30 mg kg^{-1}, but no effect on striatal 5-HT concentration following the same dosing schedule (O'Shea et al. 2001).

Both cathinone and methcathinone cause 5-HT release from synaptosomes (Gygi et al. 1997). *Ex vivo* high performance liquid chromatography (HPLC) analysis of rat brain tissue has shown a decrease in 5-HT and 5-HIAA in the prefrontal cortex, but an increase in dopamine in the nucleus accumbens following repeated administration of cathinone (Banjaw et al. 2003). An acute s.c. injection of methcathinone has no effect on striatal tissue monoamine concentrations at 30 min and 2 h post-injection, however the activity of the rate limiting enzyme in 5-HT synthesis, TPH is decreased at these time points (Gygi et al. 1996). However, in the same study repeated methcathinone did decrease striatal tissue 5-HT and its metabolite (5-HIAA) 18 h after four doses of methcathinone (30 mg kg^{-1}) at 4 h intervals. Additionally, hippocampal and frontal cortical tissue 5-HT and 5-HIAA were also decreased following the same dosing schedule and

activity of TPH and tyrosine hydroxylase (TH) were reduced in the striatum, hippocampus and frontal cortex.

Dopamine is an essential modulator of motivated behaviour associated with natural rewards, such as food. Recreational drugs which alter dopamine release or function would therefore be expected to affect this reward pathway. Indeed in addition to elevating 5-HT, MDMA also causes rapid release of dopamine after administration (Sharp et al. 1987; Crespi et al. 1997; Green et al. 2003; Benamar et al. 2008). However, unlike 5-HT, tissue concentrations of dopamine in the rat brain are raised in the few hours following MDMA administration while the levels of its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), are reduced (Stone et al. 1986). MDMA causes the release of dopamine from brain slices and prevents reuptake of dopamine into brain synaptosomes (Schmidt et al. 1987; Steele et al. 1987). Both cathinone and methcathinone cause dopamine release from synaptosomes (Kalix 1984; Kalix and Glennon 1986; Gygi et al. 1997) and *ex vivo* HPLC analysis of rat brain tissue has shown an increase in tissue dopamine in the nucleus accumbens following repeated administration of cathinone (Banjaw et al. 2003). Further evidence for cathinone acting upon the dopaminergic system comes from its anticataleptic effects in a Parkinson's disease model (Banjaw et al. 2003) where cathinone antagonises haloperidol-induced catalepsy, in a similar manner to MDMA. Methcathinone also increases striatal HVA 3 h post-injection (Gygi et al. 1996). In the same study, microdialysis was used to determine the acute effects of methcathinone (1 mg kg^{-1}) on extracellular dopamine where a 409% increase in extracellular DA was observed. Changes in 5-HT were also prevented by depletion of dopamine by striatal lesioning with 6-hydroxydopamine (6-OHDA), further suggesting the important role of dopamine in the serotonergic response to methcathinone.

Few studies have examined the effect of mephedrone on central monoamines. *In vivo* microdialysis studies showed that mephedrone rapidly increases nucleus accumbens dopamine and 5-HT levels to a greater extent than MDMA, and that release of 5-HT is also greater than dopamine (Kehr et al. 2011; Baumann et al. 2012; Wright et al. 2012).

This is in marked contrast to MDMA which causes a much greater release of dopamine than 5-HT in the same region (O'Shea et al. 2005). Motbey et al., (2012) found that acute injection or 10 daily injections of 30 mg kg^{-1} of mephedrone increased striatal and hippocampal tissue dopamine levels, but decreased 5-HT in the same regions 60 min post-injection. The decrease in 5-HT levels may reflect the greater release of 5-HT than dopamine.

1.3.3 Monoamine neurotoxicity

Administration of a single high dose or multiple doses of MDMA to rats causes long term serotonergic neurotoxicity in several brain areas (Stone et al. 1986; Battaglia et al. 1987) which is distinct from the acute initial (<24 h) 5-HT release and begins between one and seven days post-administration (Schmidt et al. 1987). The most severe depletions occur in the striatum, frontal cortex and hippocampus, and coincide with decreased binding to and uptake of radio-labelled paroxetine by SERT, as well as long term decreases in TPH activity (O'Shea et al. 2006; Xie et al. 2006). Amphetamine-induced damage to 5-HT neurons in rats is known to return to normal over time with abstinence (Scanzello et al. 1993; Lew et al. 1996). This neurotoxicity is strain dependent where Dark Agouti rats are more sensitive (single doses of 10-15 mg kg^{-1} cause greater than 50% depletion of 5-HT) compared to other strains, where multiple or high doses are required to cause the same level of 5-HT depletion (Colado et al. 1993; Aguirre et al. 1998; O'Shea et al. 1998; Shankaran and Gudelsky 1999). In contrast, MDMA administration causes long term dopamine depletion in mice, without any accompanying 5-HT loss (Stone et al. 1987; Logan et al. 1988).

MDMA neurotoxicity is observed following systemic administration but not central administration, suggesting that peripheral metabolism is required for neurotoxic damage to occur (Paris and Cunningham 1992). The exact mechanism of MDMA neurotoxicity is unknown, but there is a close relationship between MDMA-induced hyperthermia and neurotoxicity (Malberg and Seiden 1998) whereby pharmacological interventions and

other manipulations which prevent hyperthermia, such as reduced room temperature, also prevent neurotoxicity (Farfel and Seiden 1995; Malberg et al. 1996; Sprague et al. 2003; Johnson and Yamamoto 2010; Granado et al. 2011). MDMA-induced hyperthermia also promotes free radical formation which is thought to contribute to neurotoxicity, and 5-HT metabolism in an oxidising environment also leads to the formation of toxic metabolites (Wrona et al. 1995; Colado et al. 1999). However, repeated low doses of MDMA can cause long term neurotoxicity without any coinciding hyperthermic response (O'Shea et al. 1998). MDMA may also induce neurotoxicity by increasing intracellular monoamine neurotransmitter release thereby increasing MAO activity which causes the formation of H_2O_2 which can be converted to a hydroxyl radical, resulting in further oxidative stress-related damage (Alves et al. 2007). Furthermore, lesioning of dopamine neurons in the brain by 6-OHDA prevents MDMA-induced neurotoxicity while pre-treatment with the dopamine precursor L-DOPA enhances neurotoxicity (Schmidt et al. 1990).

There is little published data on the neurotoxic effects of cathinone but chronic administration of 100 mg kg^{-1} to rats does result in dopamine depletion (Feyissa and Kelly 2008). Methcathinone is available in a racemic mixture but is mostly consumed in the S(-) enantiomer with small amounts of R(+)-methcathinone. Sparago et al. (1996) identified that in the mouse both enantiomers have a neurotoxic effect on striatal dopamine with the R(+) form producing greater depletion than the S(-) form. Similar results were observed in the rat, where a dose of 50 mg kg^{-1} of either enantiomer depleted striatal dopamine however the S(-) enantiomer was found to cause the greatest decrease. Additionally, in the same study, while methcathinone had no effect on 5-HT and 5-HIAA in mice, S(-)-methcathinone depleted 5-HT and 5-HIAA in the hippocampus and cortex of rats following 50 mg kg^{-1}. Repeated methcathinone (4 x 30 mg kg^{-1}) has also been shown to cause depletion of 5-HT, dopamine and their metabolites in the rat striatum 30 days after the last injection (Gygi et al. 1997). Importantly, neurotoxicity is not observed following methcathinone at a dose required to produce behavioural changes, much higher doses are needed for neurotoxicity (Gygi et al. 1996).

The development of neurotoxicity following mephedrone administration is more controversial. To date, one study identified neurotoxic loss of hippocampal 5-HT seven days after 4 x 10 or 25 mg kg^{-1}, s.c. in rats, where loss of SERT was also reported (Hadlock et al. 2011). Subsequent studies using repeated doing schedules (4 x 3 or 10 mg kg^{-1} at 2 h intervals or 30 mg kg^{-1} twice daily for four days) found no neurotoxic loss of 5-HT, dopamine or their metabolites two weeks after the final injection (Baumann et al. 2012; den Hollander et al. 2013). Once daily injections of 7.5, 15 or 30 mg kg^{-1} for ten days also failed to produce any decrease in 5-HT or dopamine levels in the striatum or hippocampus (Motbey et al. 2012). One study in mice has demonstrated dopamine and 5-HT neurotoxicity seven days after 4 x 50 mg kg^{-1}, s.c., but only transient dopamine depletion (3 days post-injection) was observed after 4 x 25 mg kg^{-1} (Martinez-Clemente et al. 2014). However, other studies in mice failed to detect any mephedrone-induced neurotoxic loss of striatal dopamine terminal integrity seven days after 4 x 40 mg kg^{-1} (Angoa-Perez et al. 2012) or 30 mg kg^{-1} twice daily for four days (den Hollander et al. 2013) and a single i.p. injection of mephedrone (20 mg kg^{-1}) also fails to produce hippocampal 5-HT depletions two days after administration (Angoa-Perez et al. 2014).

1.4 Preclinical studies

1.4.1 Locomotor effects and stereotypical behaviour

The serotonin syndrome includes various behaviours associated with 5-HT releasing compounds, such as hyperactivity, head weaving, piloerection, protrusion of the eye, fore-paw treading, salivation, defecation, penile erection and ejaculation. MDMA produces dose-dependent hyperactivity along with the other symptoms of the serotonin syndrome (Callaway et al. 1990; Fone et al. 2002; Bull et al. 2004). It is attenuated by administration of 5-HT re-uptake inhibitors and 5-HT receptor antagonists (Callaway et al. 1990; Kehne et al. 1996; Bankson and Cunningham 2002), as well as by bilateral 6-OHDA lesioning of the nucleus accumbens and dopamine receptor antagonist administration, thereby implicating both

5-HT and dopamine in this hyperactivity (Gold et al. 1989; Ball et al. 2003; Bubar et al. 2004). Rapid repeat injections of MDMA cause sensitisation to the locomotor stimulant effects of this amphetamine (Rodsiri et al. 2011).

Since it is thought that cathinone has a similar pharmacological profile to amphetamine it is not surprising that they both produce similar stereotypical behaviours. For example, cathinone increases locomotor activity, rearing and active sitting at low doses in rats (Glennon et al. 1987; Banjaw et al. 2003; Banjaw et al. 2005) as well as wing extension, loss of righting reflex, distress vocalisation and flat body posture in chicks (Bronson et al. 1995). Methcathinone also produces hyperactivity in mice and rats (Glennon et al. 1987; Glennon et al. 1995) and is much more potent than cathinone in producing locomotor hyperactivity in mice (Glennon et al. 1987). These behavioural changes occur at doses much lower than those required to produce neurotoxicity (Gygi et al. 1996).

Numerous studies have shown that mephedrone induces hyperactivity in rodents. Considering its short plasma half-life, it is unsurprising that the locomotor response to mephedrone is short in duration following intraperitoneal (i.p.) injection; however a more sustained hyperactivity profile is observed with oral dosing (Kehr et al. 2011; Martinez-Clemente et al. 2013). Sensitisation to mephedrone-induced hyperactivity has been observed when measured ten days after 0.5 mg kg^{-1} once daily for five days or following seven daily injections of 15 mg kg^{-1} (Lisek et al. 2012; Gregg et al. 2013), but not after 10 daily injections of 30 mg kg^{-1} (Motbey et al. 2012).

1.4.2 Body temperature

MDMA can cause hyperthermia in humans and this is considered one of its most harmful adverse effects. However, MDMA-induced changes in body temperature are more complicated in rats where they depend on a number of factors such as room temperature, whether the animals are individually or group-housed, housing conditions (such as bedding type) and dose

(Green et al. 2005). At normal ambient temperatures (20-22 °C) MDMA generally causes hyperthermia (+1-2 °C), however hypothermia has been reported in a number of studies. Typically, group housing, high ambient temperature (>25 °C) and administration of repeated doses or a high dose of MDMA will cause hyperthermia in rats, where as individual housing, low ambient temperature and acute low dose of MDMA will cause hypothermia (Gordon et al. 1991; Colado et al. 1993; Dafters 1994; Rodsiri et al. 2011). Additionally, rapid repeated dosing of MDMA causes a biphasic temperature response where initial hypothermia is converted to hyperthermia after the third injection (Rodsiri et al. 2011).

In rats, dopamine D_1 receptors are involved in the hyperthermic response to MDMA, while D_2 receptors are involved in MDMA-induced hypothermia (Mechan et al. 2002; Green et al. 2005). Activation of α_1-adrenoceptors causes peripheral vasoconstriction thereby reducing blood flow to prevent heat loss, while α_2-adrenoceptor activation is involved in vasodilatation causing increased blood flow to promote heat loss and β-adrenoceptor activation in brown adipose tissue promotes heat generation. In rats, combined α_1 and β-adrenoceptor blockade attenuates MDMA-induced hyperthermia (Sprague et al. 2004). Where rats display a biphasic temperature response to MDMA, blockade of α_{2A}-adrenoceptors prolongs the initial hypothermia induced by MDMA so that the subsequent transition to hyperthermia is not observed within 300 min post-injection (Bexis and Docherty 2006). In mice, administration of an α_1- or α_{2A}-adrenoceptor antagonist prevents an initial MDMA-induced hyperthermia, causing a biphasic temperature response to this amphetamine where hypothermia is first observed followed by hyperthermia. Collectively, this evidence suggests a role of the noradrenergic system in MDMA-induced changes to thermoregulation.

Direct action of MDMA on 5-HT release is not involved in the temperature response to MDMA but 5-HT receptor activation is involved in modulating the hyperthermic response. MDMA-induced hyperthermia is closely correlated with 5-HT neurotoxicity such that the severity of neurotoxicity is associated with the level of hyperthermia induced where ambient room temperatures above 26 °C (1-2 °C change in core body temperature)

caused greater 5-HT depletion (Malberg and Seiden 1998). Equally, 5-HT depletion by prior administration of a neurotoxic dose of MDMA causes a disruption in thermoregulation at high ambient temperature and drugs that protect against MDMA-induced hyperthermia also prevent 5-HT neurotoxicity (Malberg et al. 1996; Mechan et al. 2001). Importantly, increased room temperature does not cause 5-HT, dopamine or noradrenaline depletion in saline treated rats.

Cathinone and methcathinone reportedly cause hyperthermia in humans (Emerson and Cisek 1993; Kelly 2011) and mephedrone users have reported uncontrollable changes in body temperature, with cold or blue fingers being among the most commonly reported adverse effects (Schifano et al. 2011; Winstock et al. 2011). Although there is limited preclinical data available on the acute effects of these compounds, some studies have shown cathinone- and methcathinone- induced hyperthermia in rabbits and rats (Kalix 1980; Tariq et al. 1989; Rockhold et al. 1997). Additionally, acute s.c. mephedrone injection causes hypothermia in rats (Wright et al. 2012; Miller et al. 2013), while repeated injection has been shown to cause hyperthermia at normal ambient temperature (Baumann et al. 2012) or at a room temperature of 27 °C (Hadlock et al. 2011).

1.4.3 Cardiovascular effects

MDMA has a high affinity for adrenoceptors which regulate peripheral blood flow by promoting vasoconstriction or dilatation. In rats, MDMA causes tachycardia, arrhythmia and vasoconstriction, as indicated by decreased tail temperature (Gordon et al. 1991; Badon et al. 2002) and evidence suggests that combined adrenoceptor and 5-HT receptor actions of MDMA are involved in the peripheral vasoconstriction effects of MDMA (McDaid and Docherty 2001).

The cathinones elicit tachycardia in human users (section 1.4), however there are few preclinical studies investigating the cardiovascular effects of these compounds. Cathinone induces vasoconstriction of rabbit perfused

ear artery by electrical field stimulation, as well as causing direct vasoconstriction of guinea pig aortic rings, coronary vasoconstriction in guinea pig isolated Langendorff hearts and coronary arteries (Broadley 2010). In rats, mephedrone also causes a dose-dependent increase in arterial pressure and tachycardia (Meng et al. 2012; Varner et al. 2013). The arterial pressure response is attenuated by the non-selective α-adrenoceptor antagonist, phentolamine, suggesting that peripheral activation of α-adrenoceptors is important in eliciting this response, while mephedrone-induced tachycardia was blocked by the β_1-adrenoceptor antagonist, atenolol (Varner et al. 2013). It is likely that mephedrone is causing these responses by acting as a substrate at NET to release noradrenaline from cytoplasmic stores as it causes pressor responses and tachycardia in reserpine pre-treated rats making it unlikely that it is eliciting its cardiovascular effects by noradrenaline release from vesicular stores in peripheral sympathetic nerve terminals (Meng et al. 2012; Varner et al. 2013).

1.4.4 Cognitive tests

The term cognition is used to describe the process of learning and memory. Learning is classified as either associative (forming a connection between two stimuli) or non-associative (by repeated exposure to a stimulus). There are also several subtypes of memory, these being broadly categorised as sensory, short-term (or working memory) and long-term memory. The effect of MDMA on learning and memory in animal models is controversial as results vary depending on the task, dose and dose schedule used between studies. The novel object discrimination (NOD) task utilises the rodent's natural instinct to explore a novel object as an indicator of non-spatial visual recognition memory without the need for training or reinforcement (Ennaceur and Delacour 1988). MDMA disrupts NOD in a dose related manner, such that single administration of 10 mg kg^{-1} to mice did not alter the preference for the novel object in the choice trial one day post-injection, whereas mice that had received repeated daily administrations (for seven days) were unable to discriminate novel from familiar objects (Nawata et al. 2010). NOD was impaired further when it was measured seven days after the final repeated injection, which may be

attributed to the long term neurotoxic effects of MDMA already discussed (section 1.3.1.3). Assessment of NOD in rats have shown varied results where acute MDMA administration caused either reduced exploration in the familiarisation trial, repeated MDMA (4 x 5 mg kg^{-1} over 4 h on two consecutive days, i.p.) disrupted NOD after a 15 min but not 60 min ITI, while rapid repeated MDMA (3 x 5.6 mg kg^{-1} over 4 h, i.p.) disrupts NOD 14 days post-injection (Morley et al. 2001; Camarasa et al. 2008; Rodsiri et al. 2011). In contrast, there is conclusive evidence that both acute and repeated MDMA administration disrupts spatial visual recognition memory in water maze tasks (Camarasa et al. 2008; Skelton et al. 2008; Vorhees et al. 2009).

There are few studies investigating the effects of the cathinones on cognition. Acute mephedrone improves visual spatial memory and learning, but not spatial working memory in rhesus macaques (Wright et al. 2012). Mephedrone also disrupts NOD 36 days following once daily administration of 30 mg kg^{-1} to rats for 10 days (Motbey et al. 2012). Additionally, the same dose administered twice daily to mice over four days reduces working memory on the T-maze when measured three weeks post-administration (den Hollander et al. 2013), but there was no effect on spatial visual recognition memory (as measured by the Morris water maze task) in the same animals five weeks after the final mephedrone injection.

Pre-pulse inhibition of acoustic startle (PPI) is a behavioural test which uses sound as the startling stimulus to measure sensorimotor gating. Acute MDMA injection reduces auditory and visual PPI in rats (Kehne et al. 1996; Vollenweider et al. 1999). In contrast, repeated administration of cathinone reduces PPI in rats at a dose of 1.5 mg kg^{-1}, but acute cathinone has no affect on PPI (Banjaw et al. 2005).

1.4.5 Anxiety behaviour

The elevated plus maze is used to assess anxiety-related behaviour in rodents where time spent in the more aversive open arms, the number of

head dips over the side of the open arms and stretch attends are used as indicators of 'anxiety-related' behaviour (Pellow et al. 1985). MDMA causes a dose-related decrease in elevated plus maze behaviours (Ho et al. 2004) where administration of low to medium doses (<10 mg kg^{-1}) cause increased avoidance of the open arms and reduced exploratory head dips, but this effect is less marked with higher doses. Previous repeated exposure to MDMA (4 x 5 mg kg^{-1} on two consecutive days) followed by eight weeks of abstinence did not have any effect on anxiety related behaviour (Bull et al. 2004). Repeated mephedrone administration does not cause any change in anxiety-related behaviour when rats were tested 11 days after the last of ten injections of 30 mg kg^{-1}, or 14 days following twice daily injections of 30 mg kg^{-1} for four days (Motbey et al. 2012; den Hollander et al. 2013). The effects of cathinone and methcathinone on elevated plus maze behaviour have not been evaluated.

1.4.6 Drug discrimination studies

In drug discrimination studies, subjects are typically trained to distinguish a particular dose of a rewarding drug from non-rewarding vehicle (most common), a different dose of the same drug or a different training drug using a two-lever operant chamber (Glennon et al. 1983; Young 2009). MDMA substitutes for cathinone and amphetamine in rats but amphetamine does not substitute for MDMA in rats trained to discriminate MDMA from saline (Schechter 1987; Oberlender and Nichols 1988; Glennon 1991). Additionally, MDMA partially substitutes for cocaine (Broadbent et al. 1989). It has also been demonstrated that methcathinone elicits amphetamine lever responding in rats, at a dose of 0.5-1.0mg kg^{-1}, and cocaine lever responding in rats, at doses of 0.25-0.75 mg kg^{-1} (Schechter, 1997; Young and Glennon, 1993; Glennon et al, 1987). Baboons that were previously treated to self-inject cocaine were also observed to frequently self-inject methcathinone (Kaminski and Griffiths, 1994).

1.5 Effects in humans

MDMA has a range of desirable effects in users including euphoria and empathy, but these can be accompanied by adverse effects such as tachycardia, nausea, tremors, jaw clenching, insomnia, numbness, anxiety, fear, paranoia, headache and uncomfortable changes in body temperature (Baylen and Rosenberg 2006). Severe hyperthermia is considered among the most harmful adverse effects of MDMA as it is the most frequent and can cause other problems to arise such as dehydration, the breakdown of muscle fibres and increased water consumption which can lead to water intoxication and death (Chadwick et al. 1991; Campkin and Davies 1992; Kalant 2001; Hall and Henry 2006). Studies investigating the role of different neurotransmitter systems on the effects of MDMA in humans have suggested that the overall psychological effects of MDMA, such as positive mood, thought disturbances, self-confidence and reduced control over thought and body are related to 5-HT release, while the euphoric effects may be partially related to dopamine (Liechti and Vollenweider 2001). In these studies the subjective effects of MDMA began 30-60 min post-administration and last for approximately 3.5 h.

Frequent MDMA use is associated with the development of tolerance, with several studies indicating a decline in effect with increased use, as well as an increase in dosing amongst users (Parrott 2002). MDMA can cause impairment on word list learning of one or two words out of a total of 20 words, which is similar to that observed in a person who has a blood alcohol concentration of 80mg $100ml^{-1}$ (the maximum legal level for driving; ACMD, 2008). Users of mephedrone have claimed that it is similar to MDMA in its capability to produce feelings of euphoria and empathy; however, recent reports from emergency rooms and individuals have revealed that mephedrone may be similar to MDMA in its adverse effects also. However, care must be taken in interpreting these user reports as MDMA and mephedrone can be mis-sold as each other or different compounds. For a comparison of some of the reported clinical effects of mephedrone, MDMA, cathinone and methcathinone see Tables 1.2 and 1.3.

Table 1.2 Comparison of the desired effects of the cathinones and MDMA.

Reported effects	MDMA	Mephedrone	Cathinone	Methcathinone
Euphoria	✓	✓	✓	
Increased energy	✓	✓	✓	
Increased alertness	✓	✓	✓	
Excitation			✓	✓
Increased sociability	✓	✓		
Calm	✓			
Sexual arousal	✓		✓	
Analgesia			✓	
Sharpened senses	✓			

Data taken from Feyissa and Kelly, (2008); Kalant, (2001); Kelly, (2011); Newcombe, (2009); Emersen and Cisek, (1993).

Table 1.3 Comparison of the adverse effects of the cathinones and MDMA.

Reported effects	MDMA	Mephedrone	Cathinone	Methcathinone
Bruxism	✓	✓	✓	
Temperature changes	✓	✓	✓	✓
Fatigue	✓			
Tachycardia	✓	✓	✓	✓
Hypertension	✓		✓	
Cold/blue Fingers		✓		
Sweating	✓	✓		
Dry mouth	✓	✓	✓	
Nausea/vomiting	✓	✓		✓
Difficulty urinating			✓	
Headache	✓		✓	✓
Muscle ache	✓			
Dizziness	✓	✓	✓	
Numbness/tingling	✓			✓
Tremors	✓		✓	✓
Agitation	✓	✓	✓	✓
Confusion		✓		✓
Cognitive impairment	✓	✓	✓	
Insomnia	✓	✓	✓	✓
Changes in appetite	✓	✓	✓	✓
Anxiety	✓	✓	✓	✓
Anhedonia	✓	✓	✓	
Fear/paranoia	✓	✓	✓	✓
Hallucinations	✓	✓	✓	✓
Skin rash		✓		
Blurred vision	✓	✓		
Nose/throat bleeds		✓		

Data taken from Baylan and Rosenberg (2006); Cox and Rampes (2003); Dargan et al. (2010); Freeman et al. (2012), Feyissa and Kelly (2008); Kalant (2001); Kelly (2011); Newcombe (2009); Emersen and Cisek (1993).

Although little is known about the long term toxic effects of mephedrone, adverse events, such as, headaches, nausea/vomiting, nose bleeds, tachycardia, hypertension, seizures and uncomfortable changes in body temperature (sweating, cold/blue fingers), amongst others, have been reported in mephedrone users and in the UK it has been implicated as cause of death of a number of young people (ACMD 2010). For some of these individuals it is difficult to infer that mephedrone is the sole cause of death, as toxicological tests have found other stimulants, such as cocaine, post-mortem suggesting that the cause of death may have been polydrug toxicity (Gerace et al. 2014). Corkery et al. (2012) compiled available data on suspected mephedrone-related deaths in the UK up until August 2011. Of the 125 deaths reported to be as a result of mephedrone use, 87 have tested positive for mephedrone post-mortem, and 13 were awaiting toxicological confirmation at the time of publication. Mephedrone toxicity was mentioned as cause of death in 24 of these cases, and polydrug toxicity in a further ten cases. Therefore studies are needed that will provide evidence as to the direct effects of mephedrone on neurotransmitter systems as well as its physiological and behavioural effects.

1.6 Experimental approach

Due to its substantial media attention, implication in a number of deaths in the UK in recent years and limited published pharmacological data, mephedrone is the main focus of the work presented in this thesis. At the commencement of this research there were no published data on the effects of mephedrone in preclinical animal models, or on its mechanisms of action or adverse effects. Therefore, due to the reported subjective similarity of mephedrone to MDMA by users and the similar chemical structure to cathinones, initial studies compared the effect of mephedrone to that of these amphetamines in the rat. The known effect of MDMA in the rat was used as a source of reference to design experiments and select drug doses in these studies.

Animal models are an important asset in studying the effects of recreational drugs as the high risk of toxicity limits the ability of researchers to study the effects of these drugs in humans. Both mice and rats are commonly used in such studies. Rats were used in the current set of studies as their neurochemical response to MDMA more closely resembles that observed in humans, compared to mice. There are a number of different rat strains used to assess the behavioural and neurochemical effects of MDMA. In the current study, Lister hooded rats were chosen as they have better eyesight than albino strains for NOD studies and were therefore used in all studies for consistency and comparison with selected previous studies with MDMA (Rodsiri et al. 2011). Given the differences in the pharmacokinetic profile of MDMA in humans and rats (as discussed in section 1.2), it is important to select doses used in preclinical models that are of translational value to recreationally relevant doses. A dose of 4 mg kg^{-1} of MDMA in the rat is comparable with a user taking approximately one ecstasy tablet containing 70 mg of MDMA (Green et al. 2012). However, the half-life of MDMA is much shorter in rats than in humans, so a higher dose of 10 mg kg^{-1} was also administered to provide a longer duration of action of MDMA (Baumann et al. 2009). Using allometric scaling, this dose would equate to a human taking approximately 455 mg of MDMA. While caution should be taken in extrapolating animal data to humans, the work presented in this thesis can be used as a starting point for further human-based research.

Changes in body temperature are amongst the more harmful adverse effects of MDMA and, in rats, these changes are sensitive to environmental variations (section 1.5.1.2). Therefore, the studies presented in Chapter 2 investigated the effects of mephedrone, cathinone and methcathinone on rectal and tail temperature in individually housed rats in comparison to MDMA. *Ex vivo* tissue monoamine levels in the striatum, hippocampus and frontal cortex were also measured at the end of recording as MDMA is known to cause monoamine release in these regions. *Ex vivo* tissue monoamine levels in the hypothalamus were also examined due to its role in temperature regulation. To further compare mephedrone and MDMA, these parameters were also measured in group housed rats. To decipher the mechanism of action of mephedrone-induced temperature changes, the effects of dopamine and noradrenaline antagonist pre-treatments were

also measured, as well as plasma noradrenaline and adrenaline levels after mephedrone injection to get an index of any sympathomimetic actions.

In Chapter 3, the effects of chronic intermittent mephedrone on behaviour were investigated, in comparison to MDMA and cathinone. MDMA causes hyperactivity, changes in cognition and sensorimotor gating, as well as long term neurotransmitter changes (sections 1.3 and 1.5). Therefore locomotor activity (LMA), NOD, conditioned emotional response (CER), prepulse inhibition to acoustic startle response were assessed, as well as *ex vivo* monoamine levels in specific brain regions seven days post-injection. Locomotor activity was assessed twice, once at the start of the study (day one) and again at the end of the study (day 16) to evaluate any drug-induced locomotor sensitisation.

In the NOD task the rat explores two identical objects in the first, familiarisation trial, followed by an inter trial interval (ITI) after which the rat is re-exposed to one of the familiar object and one novel object in the choice trial. The ability of the rat to distinguish novel from familiar objects is sensitive to pharmacological intervention and influenced by the length of the ITI. As previously discussed, MDMA disrupts NOD in a dose related manner (section 1.5.1.4). The CER task is a measurement of amygdala and hippocampal dependent associative memory. This task pairs an unconditioned stimulus (footshock) with a conditioned stimulus (context, light or tone), such that re-exposure to the non-aversive conditioned stimulus in the absence of the unconditioned stimulus will evoke a freezing behaviour that can be measured as an index of memory. Finally, pre-pulse inhibition refers to the decrease in the response to a startling stimulus when another weak stimulus precedes it closely in time. PPI is a measurement of sensorimotor gating (the brain's ability to modulate its sensitivity to incoming sensory stimuli) which is known to be inhibited in a number of disorders such as schizophrenia and autism, as well as in amphetamine users. These tasks were chosen as they can be performed without prior training and are known to be disrupted by MDMA administration.

Mephedrone users are likely to have previously taken MDMA and caffeine is known to exacerbate MDMA toxicity and is often taken concomitantly with other psychostimulants (Brandt et al. 2010; Carhart-Harris et al. 2011; Vanattou-Saifoudine et al. 2012; Rosenauer et al. 2013). Therefore, Chapter 4 investigated the effects of MDMA pre-treatment or concomitant caffeine administration on chronic intermittent mephedrone administration. A preliminary dose-response study was conducted where rats received 10 or 30 mg kg^{-1} of mephedrone and LMA was recorded for 60 min, at the end of which a single rectal temperature recording was made. This was conducted to ensure the selection of a submaximal mephedrone dose in subsequent studies. In the following two studies presented in this chapter, rats received pre-treatment of MDMA or concomitant caffeine administration and again locomotor activity, novel object discrimination, rectal and tail temperature, prepulse inhibition to acoustic startle response and *ex vivo* monoamine levels in specific brain regions were evaluated, in addition to assessment of 'anxiety-related' behaviour on the elevated plus maze. The elevated plus maze is used to assess anxiety-related behaviour in rodents where time spent in the open arms, the number of head dips over the side of the open arms and stretch attends are used as indicators of anxiety-related behaviour (Pellow et al. 1985). Anxiogenic drugs enhance avoidance of the open arms, reduced number of head dips and stretch attends, while anxiolytic drugs cause the opposite of these behaviours.

Rapid repeated dosing of MDMA causes locomotor sensitisation as well as converting MDMA-induced hypothermia into a hyperthermic response (Rodsiri et al. 2011). Therefore, Chapter 5 investigated the effects of rapid repeat injection of mephedrone on locomotor activity and core body temperature, as measured by radiotelemetry, and used *in vivo* microdialysis to examine the effect of the same dosing schedule on dopamine levels within the striatum. To further determine the role of 5-HT and dopamine in the hyperlocomotor and hypothermic response to mephedrone, bilateral intracerebroventricular (i.c.v.) injections of 5,7-dihydroxytrptamine (5,7-DHT) and 6-OHDA to deplete brain 5-HT and dopamine, respectively, were administered to rats with radiotelemetry implants for continual recording of locomotion and body temperature. In a final study rats were pre-treated with 5-HT_{1A} and 5-HT_7 antagonists to

determine the role of these receptors in mephedrone-induced changes in rectal and tail temperature.

Chapter 2 Acute effects of the cathinones and MDMA on body temperature and neurochemistry

2.1 Introduction

Severe hyperthermia is considered among the most harmful adverse effects of recreational MDMA use as it can lead to potentially fatal complications such as rhabdomyolysis, renal and liver failure and myoglobinuria (see Docherty and Green, 2010). MDMA-induced changes in body temperature are more complicated in rats and appear to depend on a number of factors such as room temperature, whether rats are individually or group-housed, housing conditions (such as sawdust on the floor) and dose (Green et al. 2005). Typically, with group housing, high ambient temperature (>25 °C) and/or administration of high or repeated doses of MDMA tend to produce hyperthermia in rats, where as individual housing, low ambient temperature and acute low dose of MDMA result in hypothermia (Gordon et al. 1991; Colado et al. 1993; Dafters 1994; Rodsiri et al. 2011).

Cathinone and methcathinone reportedly cause hyperthermia in humans (Emerson and Cisek 1993; Kelly 2011) and mephedrone users have reported uncomfortable changes in body temperature, with cold or blue fingers being amongst the most commonly reported adverse effects (Schifano et al. 2011; Winstock et al. 2011). Although there is limited preclinical data available on the acute effects of these compounds, some studies have shown cathinone- and methcathinone-induced hyperthermia in rabbits and rats (Kalix 1980; Tariq et al. 1989; Rockhold et al. 1997). Additionally, acute mephedrone injection causes hypothermia in rats (Wright et al. 2012; Miller et al. 2013), while repeated injection at 2 h intervals over 4 or 6 h has been shown to cause hyperthermia at both normal (Baumann et al. 2012) and elevated (27 °C) ambient room temperature (Hadlock et al. 2011).

In the first study in this chapter, in order to examine the potential similarities or differences between the cathinones and MDMA, rats received a single intraperitoneal (i.p.) injection of MDMA, mephedrone, cathinone or methcathinone at doses of 4 or 10 mg kg^{-1} with measurement of rectal and tail temperature for 2 h post-injection. Rectal temperature was measured

to monitor core body temperature and tail temperature was measured as an indication of changes in peripheral heat loss or conservation. At the time this study was performed there was very little comparative pharmacokinetic information mephedrone in rats and humans, and since the cathinones used in this study have similar molecular weights, the selected doses were based in part on doses relevant to those taken by MDMA users. A dose of 4 mg kg^{-1} of MDMA in the rat produces comparable plasma levels to those seen in a user taking approximately 70 mg of MDMA (Green et al. 2012). However, the half-life of MDMA is much shorter in rats than in humans, so a higher dose of 10 mg kg^{-1} was also included to provide a longer duration of action in the rat (Baumann et al. 2009). Using allometric scaling, this dose would equate to a human taking approximately 455 mg of MDMA.

In the rat, tissue levels of 5-HT are typically lowered in the first few hours following MDMA administration, while dopamine levels are raised (see section 1.3.1). In the current study, *ex vivo* tissue levels of dopamine, 5-HT and their metabolites were measured in the frontal cortex, hippocampus, striatum and hypothalamus 2 h post-injection by HPLC-ED since MDMA is a potent releaser of these monoamines in the selected regions and the hypothalamus in particular is an important brain region for regulating body temperature (Colado and Green 1994; Gudelsky and Nash 1996; Mechan et al. 2002).

As mephedrone is the most common synthetic cathinone to be taken by users it became the focus of further temperature studies. In the second study, the possible mechanisms of action for mephedrone-induced body temperature changes were investigated by pre-treating rats with compounds known to alter the temperature response to MDMA. In rats, MDMA-induced hyperthermia is completely abolished by the D_1 dopamine receptor antagonist SCH 23390 (2 mg kg^{-1}) and attenuated by the α_1-adrenoceptor antagonist Prazosin (0.2 mg kg^{-1}) (Mechan et al. 2002; Sprague et al. 2003). MDMA-induced hypothermia in rats is blocked by the D_2 dopamine receptor antagonist remoxipride (Green et al. 2005), and prolonged by the α_2-adrenoceptor antagonist BRL 44408 (1 mg kg^{-1}), (Bexis and Docherty 2006). Plasma catecholamine levels were also

measured at the end of this experiment as an indication of the effects of mephedrone on the sympathetic nervous system regulation of peripheral vascular tone and adrenal medullary function which could influence thermoregulation.

The final study in this chapter investigated the effect of group housing on mephedrone-induced changes in rectal and tail temperature (again with measurement of plasma catecholamines 2 h post-injection) since group housing is known to exacerbate MDMA-induced hyperthermia and convert MDMA-induced hypothermia to hyperthermia (Docherty and Green 2010), and since these compounds (particularly mephedrone and MDMA) are more likely to be taken in crowded environments. Since the predominant effect of mephedrone in rats is hypothermia, this study was conducted to examine whether mephedrone would increase body temperature in conditions more similar to recreational users (i.e. crowded night clubs). The temperature response to mephedrone in increased ambient temperature conditions was not examined as studies have shown that increased ambient temperature fails to produce hyperthermia in response to mephedrone (Wright et al. 2012; Miller et al. 2013).

2.2 Aims

The aims of this set of experiments were to:

(1) compare the effects of cathinone, methcathinone and mephedrone on rectal and tail temperature and *ex vivo* brain tissue monoamine levels to those of MDMA in individually-housed rats;

(2) characterise the role of α_1- and α_2- adrenoceptors and dopamine D_1 and D_2 receptors in mephedrone-induced changes in rectal temperature and plasma catecholamine levels;

(3) identify the effect of group housing on mephedrone-induced changes in rectal temperature and plasma catecholamine levels.

2.3 Materials & Methods

2.3.1 Animals

All experiments used experimentally naïve adult male Lister hooded rats (190-390 g) obtained from Charles River UK or University of Nottingham BioSupport Unit (BSU). Rats were housed in groups of 3-4 on a 12 h light-dark cycle (lights on at 07.00 h) in wire top cages (56 x 38 x 22 cm) lined with sawdust, and with cardboard tubes and wooden blocks for environmental enrichment. Ambient temperature 21±2°C and relative humidity 55±10% were constant and food (Beekay rat and mouse diet, B & K Universal Ltd, UK) and water were freely available. All temperature experiments were conducted during the light phase between 09.00 and 14.00 h. Rats were assigned to treatment groups in a pseudorandom order, while ensuring that weight distribution between groups was even. The doses of all drugs used were selected to comply with the three R's of humane animal testing and by reference to previous publications. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986, and ARRIVE guidelines with approval from the University of Nottingham Local Ethical Committee.

2.3.2 Drugs

(-)-cathinone HCl, (±)-methcathinone HCl, (±)-3,4-methylenedioxymethamphetamine HCl (MDMA), prazosin HCl and SCH 23390 HCl were purchased from Sigma Aldrich (UK). (±)-mephedrone HCl was obtained from Ascent Scientific (UK) and BRL 44408 maleate was purchased from Tocris Bioscience (UK). L-741,626 was a gift from Institut de Reserches Servier (France). Cathinone, methcathinone, mephedrone and MDMA were dissolved in 0.154 M saline vehicle. All other compounds were dissolved in lactic acid and saline then adjusted to pH 6.5 with sodium hydroxide.

2.3.3 Study 1: Effect of the cathinones and MDMA on rectal and tail temperature, and neurochemistry in individually-housed rats

2.3.3.1 Temperature recording

Rats (n=6 per treatment group) were placed in individual Perspex arenas (39 x 23.5 x 24.5 cm) with wire mesh lids. An initial temperature recording was measured at -40 min to allow for acclimatisation to the procedure and prevent a stress-induced hyperthermic response that would confound data. A rectal probe (Portec Instrumentation, UK) was inserted approximately 6.5 cm, after allowing any visible faeces to pass out of the rectum. While measuring temperature, rats were gently held by the base of the tail to raise their back legs while ensuring that the front paws were still in contact with the arena floor. Tail temperature was measured by briefly placing a MicroFlo DSP Digital Laser Perfusion Monitor (Oxford Optronix, UK) against the base of the tail. Rectal and tail temperature devices were allowed to stabilise for approximately 20 s before recording temperature. Forty min after the habituation recording, baseline temperatures were taken and rats received a single i.p. injection of saline vehicle (1 ml kg^{-1}), cathinone, methcathinone, mephedrone or MDMA (4, 10 mg kg^{-1}). Temperatures were measured at 20 min intervals for the following 2 h. Temperature measurements for low and high doses were performed on different days for all compounds except cathinone.

2.3.3.2 Tissue collection and neurochemical detection by HPLC-ED

Rats were killed 2 h post-injection by concussion and immediate decapitation. Brains were rapidly removed for dissection of hypothalamus and right frontal cortex, hippocampus and striatum at 4 °C on a refrigerated table (BC72: Osborne refrigeration, UK). Tissue was snap frozen in liquid nitrogen and stored at -80 °C until analysis by high performance liquid chromatography with electrochemical detection (HPLC-ED) to measure levels of 5-HT, dopamine and their major metabolites.

HPLC-ED was performed according to previous methods (King et al. 2009). In summary, samples were sonicated for approximately 30 s in 800 µl of 0.05 M perchloric acid (PCA) containing 1 µM sodium metabisulfite then centrifuged at 17500 g at 4 °C for 20 min and the supernatant was filtered through a 0.45 µm syringe tip filter (Kinesis Ltd, UK). All samples were injected at a volume of 15 µl onto a TARGA C18 3 µM column (100 x 2.1 mm, Phenomenex) using a Perkin Elmer AS200 autosampler. Mobile phase consisting of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 8 mM potassium chloride, 0.15 mM octanesulfonic acid and 10 % methanol (pH 3.8-4) was circulated through the system at a flow rate of 0.2 ml min^{-1} via an Ultimate 3000 pump (Dionex) at a constant pressure of 1200 psi. Analytes were detected using an Antec VT-03 cell with a glassy carbon 2 mm working electrode set to 0.59 V with an *in situ* Ag/AgCl ISAAC reference electrode.

Mixed standards solutions were prepared daily from a stock solution (1 x 10^{-4} M) and diluted with 0.05 M PCA to concentrations of 1 x 10^{-8}, 1 x 10^{-7} and 2 x 10^{-7} M. Standards were injected at the start of each day and then after every 12 samples (see Fig 2.1 for example calibration curves). Quantification was performed using Galaxie chromatography workstation, v 1.8 against standards.

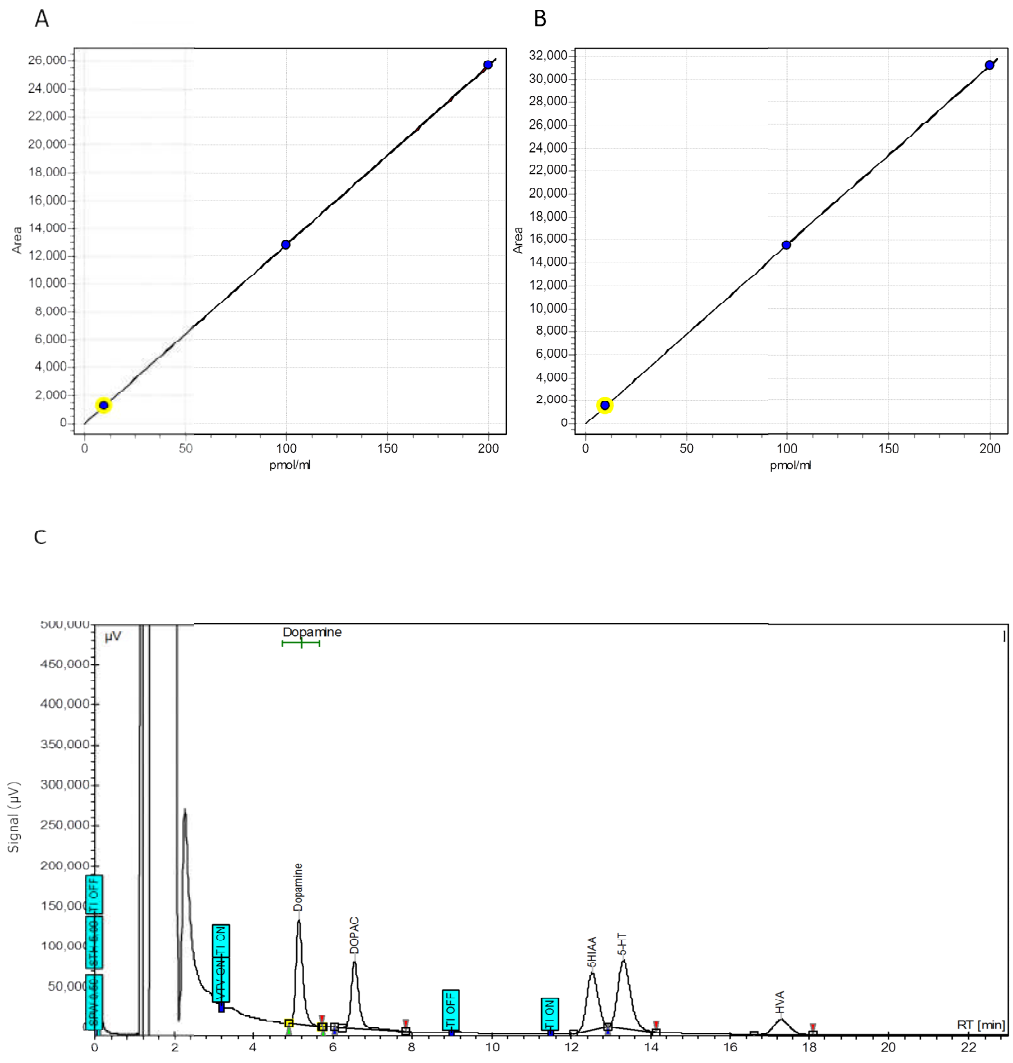


Figure 2.1 Illustrative HPLC-ED data.

Example dopamine (**A**; R^2=1.00) and 5-HT (**B**; R^2=1.00) calibration curves and a typical HPLC-ED chromatogram from an individual rat brain sample (**C**).

2.3.4 Study 2: Effect of α_1- and α_2- adrenoceptor antagonists, and dopamine D_1 and D_2 receptor antagonists on mephedrone-induced changes in rectal temperature, and plasma catecholamine levels in individually-housed rats

2.3.4.1 Temperature recording

Rats (n=6-7 per treatment group) were placed in the same arenas as described in section 2.3.3.1 and habituated to the rectal temperature measurement 40 min prior to the start of the experiment. They then received i.p. saline vehicle (1 ml kg^{-1}), prazosin HCl (0.2 mg kg^{-1}), BRL 44408 maleate (1 mg kg^{-1}), SCH 23390 HCl (2 mg kg^{-1}) or L-741,626 (0.63 mg kg^{-1}), followed 30 min later by i.p. vehicle (1 ml kg^{-1}) or mephedrone HCl (10 mg kg^{-1}). Rectal temperature was measured immediately prior to each injection and then at 20 min intervals for 2 h following vehicle mephedrone injections, as described in section 2.3.3.1.

2.3.4.2 Plasma collection and catecholamine measurements by HPLC-ED

Using a schedule 1 procedure, rats were killed by concussion and immediate decapitation 2 h post-injection. Mixed arteriovenous trunk blood was immediately collected into lithium heparin Vacutainer blood collection tubes (BD diagnostics, UK) containing 7.5 µl of 250 mM EGTA and 195 mM glutathione per ml of whole blood. Samples were centrifuged at 1000 x g for 5 min (Centaur 2: MSE Scientific Instruments, UK) and the plasma was removed, stored on ice and then at -80 °C.

Extraction and analysis were adapted from methods by Forster and MacDonald (1999). Plasma was thawed, mixed and centrifuged (1000 x g for 5 min, as previously) then 500 µl of sample was added to 100 µl of 0.05 µM 3,4-dihydroxybenxylamine HCl (internal standard, Sigma Aldrich). This was followed by the addition of 250 µl ammonia buffer (2 M ammonium chloride adjusted to pH 8.8 with 2 M ammonium hydroxide,

and containing 22 mM diphenylboric acid ethanolamine complex 13 mM disodium EDTA) and 1 ml heptanes mixture (heptane containing 6 mM tetraoctylammonium bromide and 1 % v/v octanol). This resulted in a two-layer mixture which was centrifuged again at 1000 x g for 5 min. A volume of 750 µl was removed from the organic top layer, to which 380 µl octan-1-ol and 40 µl 400 mM acetic acid were added. The sample was vortexed for 3 min (Mulit Tube Vortex Mixer: Labnet International, USA) and then centrifuged (as previously). 30-35 µl was removed from the acid droplet at the base of the tube and stored at -80 ° C. Samples were injected into a Hyperclone C18 3 µM column (100 x 2 mm, Phenomenex, UK) and detected at a potential of +0.65 V by an Antec VT-03 cell with a glassy carbon 2 mm working electrode set to 0.65 V with an *in situ* Ag/AgCl reference electrode.

The mobile phase consisted of 110 mM sodium acetate, 110 µM disodium EDTA, 347 µM sodium dodecyl sulphate, and 20 % methanol, adjusted to pH 5.2 with glacial acetic acid. Quantification was achieved manually (ChromJet Integrator: Thermo Separation Products, Waltham, MA, USA) using the internal standard to establish recovery. The mean ± SEM recovery was 80 ± 1 % and the estimated limit of detection for noradrenaline and adrenaline was 1.2-1.4 nmol L^{-1}.

2.3.5 Study 3: Effect of mephedrone on rectal and tail temperature, and plasma catecholamine levels in group-housed rats

2.3.5.1 Temperature recording

Rectal and tail temperatures were recorded as described in section 2.3.3.1, except rats remained within their home cages in groups of three throughout the procedure. Cages contained sawdust bedding as described in section 2.3.1, but environmental enrichment was removed and the cages were placed in a separate procedure room for the duration of the experiment. Rats (n=6 per treatment group) received a single i.p. injection

of saline vehicle (1 ml kg^{-1}) or mephedrone (10 mg kg^{-1}). All rats within a cage received the same treatment.

2.3.5.2 Plasma collection and measurement of catecholamine levels by HPLC-ED

Using a schedule 1 procedure, rats were killed by concussion and immediate decapitation 2 h post-injection. Plasma was collected and catecholamine levels were measured as described in section 2.3.4.2.

2.3.6 Statistical analysis

All statistical analyses were performed using GraphPad Prism v 6.02 and SPSS v 21 software. All data were checked for normality and homogeneity of variance using Shapiro-Wilk's and Levene's test, respectively. All temperature and plasma catecholamine data displayed normality and homogeneity of variance. All temperature data is presented as change from baseline (t=0 min), and statistical analyses were conducted on change from baseline, not absolute temperature. In study two, antagonists were administered at t=-30 min.

For studies one and three, which involved a single drug or vehicle injection, temperature data were analysed by two-way repeated analysis of variance (ANOVA) with treatment and time as between- and within- group factors, respectively and Bonferroni post-hoc where appropriate. The low and high doses of MDMA, mephedrone and methcathinone were administered on separate test days and since there were no between- group differences the vehicle data from each day have been pooled for clarity of presentation, however all statistical comparisons relate to the original vehicle control group on the same test day not the pooled data. Brain tissue monoamine levels and plasma catecholamine levels were analysed by one-way ANOVA with Bonferroni post-hoc test (homogeneous variance between groups) or Tamhane's post-hoc test (heterogeneous variance between groups) where appropriate. Analysis was applied separately to each neurotransmitter or

metabolite in each brain region. There was no between-group difference in vehicle data (one-way ANOVA) so for clarity of presentation, vehicle data for each region have been pooled but all statistical comparisons relate to the relevant vehicle control group.

Temperature data from study two, which involved two separate drug or vehicle injections, were analysed by three-way repeated measures ANOVA with pre-treatment (antagonist) and challenge (mephedrone) injection as between-group factors and time as the within-group factor. Plasma catecholamine levels from study three were analysed by two-way ANOVA with pre-treatment (antagonist) and challenge (mephedrone) injection as between-group factors. Bonferroni multiple comparisons post-hoc test was performed where appropriate. All data are presented as mean ± SEM and $p<0.05$ was considered significant.

2.4 Results

2.4.1 Effect of the cathinones and MDMA on rectal and tail temperature, and neurochemistry in individually-housed rats

There were no significant between-group differences in basal rectal or tail temperature immediately prior to injection, which were 40.4 ± 0.03 °C (mean ± SEM) and 29.7 ± 0.2 °C, respectively.

2.4.1.1 Rectal and tail temperature changes post-injection

MDMA and mephedrone (Fig 2.2A,B) reduced rectal temperature compared to vehicle such that there were significant main effects of treatment (4 mg kg^{-1}: $F_{(3,20)}$=24.34, p<0.001; 10 mg kg^{-1}: $F_{(2,15)}$=32.50, p<0.001) and time (4 mg kg^{-1}: $F_{(6,120)}$=20.02, p<0.001; 10 mg kg^{-1}: $F_{(6,90)}$=30.87, p<0.001) as well as a significant treatment x time interaction (4 mg kg^{-1}: $F_{(18,120)}$=13.23, p<0.001; 10 mg kg^{-1}: $F_{(12,90)}$=19.58, p<0.001). Both doses of MDMA caused sustained hypothermia that was present for the duration of testing and in the case of the high dose did not return to baseline levels within the 120 min monitoring period. Mephedrone caused a transient decrease in rectal temperature at 20 min following 4 mg kg^{-1} and from 20-40 min post-injection for the 10 mg kg^{-1} dose.

Cathinone (10 mg kg^{-1}, Fig 2.2E) caused a marked increase in rectal temperature from 40-80 min post-injection, such that there were significant main effects of treatment ($F_{(2,15)}$=8.73, p<0.01), time ($F_{(6,90)}$=9.59, p<0.001) and a treatment x time interaction ($F_{(12,90)}$=7.62, p<0.001). Treatment with 4 mg kg^{-1} of cathinone had no significant effect on rectal temperature. Similarly, methcathinone (Fig 2.2G) administered at a dose of 10 mg kg^{-1} also caused an elevation in rectal temperature from 40-120 min (treatment: $F_{(1,10)}$=18.46, p<0.01; time: $F_{(6,60)}$=4.01, p<0.01; treatment x time interaction: $F_{(6,60)}$=9.44, p<0.001), but 4 mg kg^{-1} had no effect on rectal temperature.

MDMA and mephedrone caused a significant decrease in tail temperature from 40-60 min post-injection for MDMA, and from 60-80 and 120 min post-injection for mephedrone (treatment: $F_{(2,15)}=10.29$, $p<0.01$; time: $F_{(6,90)}=3.16$, $p<0.01$; treatment x time interaction: $F_{(12,90)}=4.00$, $p<0.001$). Methcathinone (10 mg kg^{-1}) had no significant effect on tail temperature (treatment: $F_{(1,10)}=1.029$, $p>0.05$; time: $F_{(6,60)}=1.12$, $p>0.05$; treatment x time interaction: $F_{(6,60)}=0.92$, $p>0.05$). Cathinone had no significant effect on tail temperature at either dose (treatment: $F_{(2,15)}=2.43$, $p>0.05$; time: $F_{(6,90)}=1.98$, $p>0.05$; treatment x time interaction: $F_{(12,90)}=1.55$, $p>0.05$).

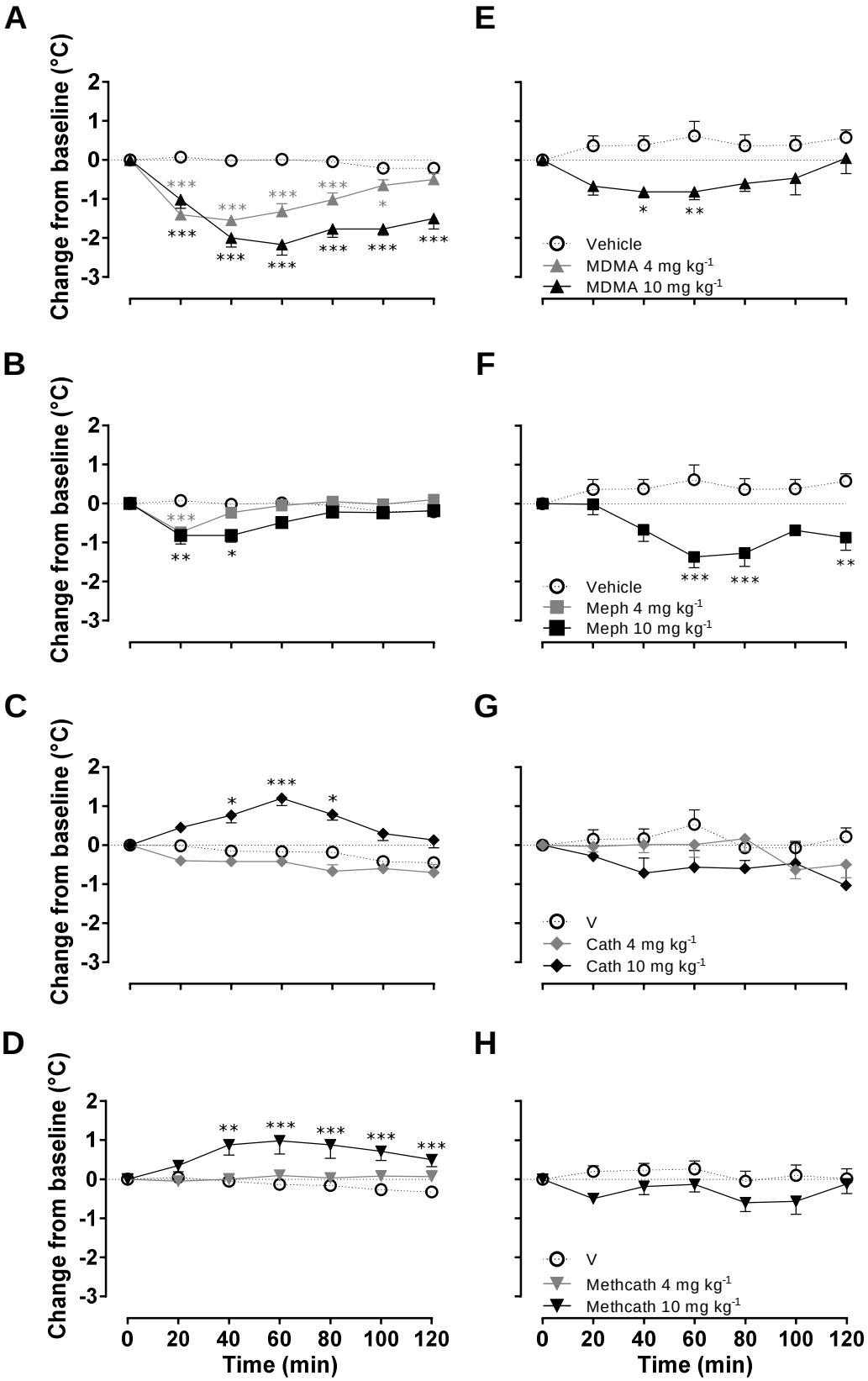

A
B
C
D
E
F
G
H
Change from baseline (°C)
Time (min)
Vehicle
MDMA 4 mg kg-1
MDMA 10 mg kg-1
Meph 4 mg kg-1
Meph 10 mg kg-1
V
Cath 4 mg kg-1
Cath 10 mg kg-1
Methcath 4 mg kg-1
Methcath 10 mg kg-1

Figure 2.2 Effects of acute MDMA, mephedrone, cathinone or methcathinone on rectal and tail temperature.

Adult male Lister hooded rats (n=5-6 per treatment group) received a single i.p. injection of saline vehicle (1 ml kg^{-1}) or (**A**, **E**) 4 or 10 mg kg^{-1} of MDMA, (**B**, **F**) mephedrone (meph), cathinone (cath; **C**, **G**) or methcathinone (methcath; **D**, **H**) at 0 min and (**A**, **B**, **C**, **D**) rectal and (**E**, **F**, **G**, **H**) tail temperatures were assessed for the following 2 h. Data are expressed as temperature change (°C, mean ± SEM) from baseline temperature (0 min). In cases where different dose levels were administered on different test days vehicle data for each compound have been pooled for clarity of presentation, after first confirming no significant difference between each vehicle group. All statistical analysis refer to the vehicle for the relevant individual test day. ***p<0.001, **p<0.01, *p<0.05 compared to saline vehicle, Bonferroni multiple comparisons post-hoc test following two-way repeated measures ANOVA.

2.4.1.2 Neurochemistry

Mephedrone had no effect on *ex vivo* tissue levels of dopamine, 5-HT or their metabolites at 2 h post-injection. MDMA had no effect on *ex vivo* dopamine levels 2 h post-injection but the 10 mg kg^{-1} dose reduced DOPAC ($F_{(2,14)}$=3.77, p<0.05, table 2.1) and HVA ($F_{(2,14)}$=5.30, p<0.05) in the striatum only. Cathinone (10 mg kg^{-1}) increased striatal dopamine ($F_{(2,15)}$=7.87, p<0.01) while both doses of cathinone increased hypothalamic DOPAC ($F_{(2,15)}$=10.02, p<0.01). The highest dose of methcathinone increased dopamine and HVA levels in the frontal cortex (dopamine: $F_{(1,10)}$=5.82, p<0.05; HVA: ($F_{(1,10)}$=6.40, p<0.05), hippocampus (dopamine: $F_{(1,10)}$=7.63, p<0.05; HVA: ($F_{(1,10)}$=11.02, p<0.01) and striatum (dopamine: $F_{(1,10)}$=4.77, p=0.05; HVA: ($F_{(1,10)}$=10.86, p<0.01).

Both doses of MDMA caused a significant reduction in hippocampal 5-HT (4 mg kg^{-1}: $F_{(3,20)}$=3.08, p=0.05; 10 mg kg^{-1}: $F_{(2,14)}$=22.66, p<0.001, table 2.2). The highest dose of MDMA also reduced levels of 5-HIAA in the hippocampus ($F_{(2,14)}$=30.27, p<0.05), hypothalamus ($F_{(2,15)}$=5.40, p<0.05) and striatum ($F_{(2,14)}$=8.48, p<0.01). In contrast the highest dose of cathinone increased 5-HT levels in the hypothalamus ($F_{(2,15)}$=5.31, p<0.05) and both 5-HT ($F_{(2,15)}$=3.69, p=0.05) and 5-HIAA ($F_{(2,15)}$=13.68, p<0.001) levels in the striatum. The highest dose of methcathinone also increased striatal 5-HIAA levels ($F_{(1,10)}$=18.31, p<0.01).

Table 2.1 Ex vivo brain tissue dopamine, DOPAC and HVA levels.

	Tissue levels (% of vehicle)			
Treatment	FCtx	Hip	Str	Hyp
	Dopamine			
MDMA 4	93.5 ± 10	98.9 ± 9	116.4 ± 49	-
MDMA 10	177.9 ± 66	122.0 ± 9	133.4 ± 7	141.2 ± 32
Meph 4	114.4 ± 24	100.9 ± 10	88.1 ± 39	-
Meph 10	114.1 ± 19	226 ± 122	85.6 ± 10	92.2 ± 12
Cath 4	73.1 ± 10	27.3 ± 8	117.2 ± 11	94.2 ± 12
Cath 10	102.9 ± 21	33.1 ± 19	145.5 ± 5**	99.7 ± 8
Methcath 4	103.7 ± 12	99.9 ± 9	111.6 ± 47	-
Methcath 10	156.3 ± 18*	192.3 ± 28*	117.4 ± 7*	78.9 ± 7
	DOPAC			
MDMA 4	101.5 ± 29	97.8 ± 9	107.5 ± 17.4	-
MDMA 10	95.2 ± 33	97.7 ± 6	61.0 ± 4*	102.3 ± 27
Meph 4	93.3 ± 20	93.4 ± 7	99.7 ± 10	-
Meph 10	102.9 ± 20	173.0 ± 46	69.5 ± 9	86.5 ± 15
Cath 4	74.1 ± 10	37.8 ± 8	106.1 ± 9	78.6 ± 5*
Cath 10	80.5 ± 10	44.8 ± 21	125.3 ± 10	69.2 ± 2**
Methcath 4	71.5 ± 9	121.8 ± 11	79.9 ± 11	-
Methcath 10	130.1 ± 10	126.8 ± 16	92.0 ± 8	84.8 ± 13
	HVA			
MDMA 4	-	-	110.8 ± 16	-
MDMA 10	174.9 ± 71	151.1 ± 43	60.9 ± 2*	93.0 ± 24
Meph 4	-	-	106.2 ± 12	-
Meph 10	166.8 ± 44	225.4 ± 38	78.3 ± 16	82.6 ± 14
Cath 4	38.6 ± 11	29.9 ± 8	105.7 ± 9	67.6 ± 7
Cath 10	71.8 ± 18	56.5 ± 15	127.6 ± 14	91.8 ± 9
Methcath 4	-	-	100.0 ± 14	-
Methcath 10	198.6 ± 30*	381.8 ± 73**	175.7 ± 18**	93.6 ± 14

Neurotransmitter and metabolite levels were measured 2 h after i.p. injection of saline vehicle (1 ml kg^{-1}), MDMA, mephedrone, cathinone or methcathinone (4 or 10 mg kg^{-1}) to individually-housed adult male Lister hooded rats (n=5-6 per treatment group). Data are expressed as mean percentage (± SEM) of the dopamine, DOPAC and HVA levels in the relevant vehicle control group. Pooled levels (pmol mg^{-1} tissue; mean ± SEM) of dopamine in vehicle controls were frontal cortex (FCtx): 0.5 ± 0.08, hippocampus (Hip): 0.4 ± 0.2, striatum (Str): 45.6 ± 7.6, hypothalamus (Hyp): 4.9 ± 0.4. These values for DOPAC were FCtx: 0.3 ±

0.07, Hip: 0.2 ± 0.07, Str: 11.4 ± 1.4, Hyp: 1.4 ± 0.1 and for HVA were FCtx: 0.9 ± 0.2, Hip: 0.5 ± 0.2, Str: 7.5 ± 0.9, Hyp: 1.0 ± 0.1. Hypothalamus was not collected in all studies. Data are presented as percent of vehicle control but statistics were performed on the raw data. **p<0.01, *p<0.05 Bonferroni post-hoc following one-way ANOVA.

Table 2.2 Ex vivo brain tissue 5-HT and 5-HIAA levels.

	Tissue levels (% of vehicle)			
Treatment	FCtx	Hip	Str	Hyp
	5-HT			
MDMA 4	130.6 ± 41	91.1 ± 7*	77.1 ± 10	-
MDMA 10	72.9 ± 39	42.0 ± 4***	85.6 ± 8	101.3 ± 24
Meph 4	149.5 ± 16	99.0 ± 14	93.3 ± 15	-
Meph 10	76.3 ± 13	99.7 ± 8	110.3 ± 9	93.7 ± 15.1
Cath 4	84.6 ± 9	93.2 ± 7	108.1 ± 6	103.6 ± 7
Cath 10	95.5 ± 17	99.3 ± 8	139.2 ± 15*	112.2 ± 5*
Methcath 4	137.6 ± 14	65.8 ± 6	103.2 ± 18	-
Methcath 10	130.5 ± 18	109.3 ± 22	104.0 ± 7	83.6 ± 11
	5-HIAA			
MDMA 4	70.8 ± 14	73.6 ± 9	144.5 ± 61	-
MDMA 10	81.1 ± 32	59.0 ± 3***	70.5 ± 4**	54.9 ± 9*
Meph 4	103.4 ± 12	187.5 ± 93	99.0 ± 36	-
Meph 10	86.6 ± 9	94.5 ± 5	95.6 ± 4	84.7 ± 12
Cath 4	89.2 ± 13	89.3 ± 4	113.7 ± 7	97.5 ± 9
Cath 10	111.4 ± 15	97.4 ± 6	161.9 ± 12***	108.2 ± 4
Methcath 4	97.3 ± 12	87.5 ± 8	133.0 ± 32	-
Methcath 10	154.1 ± 20	96.8 ± 16	132.2 ± 7**	98.8 ± 7

Neurotransmitter and metabolite levels were measured 2 h after i.p. injection of saline vehicle (V, 1 ml kg^{-1}), MDMA, mephedrone (Meph), cathinone (Cath) or methcathinone (Methcath, 4 or 10 mg kg^{-1}) to individually-housed adult male Lister hooded rats (n=5-6 per treatment group). Data are expressed as mean percentage (± SEM) of the 5-HT and 5-HIAA levels in the relevant vehicle control group. Pooled levels (pmol mg^{-1} tissue; mean ± SEM) of 5-HT in vehicle controls were frontal cortex (FCtx): 3.14 ± 0.4, hippocampus (Hip): 4.4 ± 0.5, striatum (Str): 3.5 ± 0.4, hypothalamus (Hyp): 7.3 ± 0.5. These values for 5-HIAA were FCtx: 3.2 ± 0.5, Hip: 5.6 ± 0.4, Str: 4.0 ± 0.4, Hyp: 7.6 ± 0.4. Hypothalamus was not collected for all doses. Data are presented as percent of vehicle control but statistics were performed on the raw data. ***p<0.001, **p<0.01, *p<0.05 Bonferroni post-hoc following one-way ANOVA.

2.4.2 Effect of α_1- and α_2- adrenoceptor antagonists, and dopamine D1 and D2 receptor antagonists on mephedrone-induced changes in rectal temperature, and plasma catecholamine levels in individually-housed rats

There were no significant between-group differences in basal rectal temperature immediately prior to mephedrone injection, which was 39.1 ± 0.1 °C (mean ± SEM).

2.4.2.1 Rectal temperature

Pre-treatment with the α_1-adrenoceptor antagonist prazosin, (Fig 2.3A), α_{2A}-adrenoceptor antagonist, BRL 44408 (Fig 2.3C), or dopamine D_2 receptor antagonist, L-741,626 (Fig 2.3D), did not have any significant influence on temperature in rats which received saline vehicle 30 min later. Pre-treatment with the dopamine D_1 receptor antagonist, SCH 23390 (Fig 2.3B), decreased rectal temperature at a single time point 100 min after subsequent vehicle administration compared to vehicle pre-treated rats.

Mephedrone (10 mg kg^{-1}) caused a transient decrease in rectal temperature in vehicle pre-treated rats, which was evident at 10 min post-injection and is consistent with the findings of experiment 1 (Fig 2.2B). Pre-treatment with prazosin prolonged mephedrone-induced hypothermia for 60 min, for an additional 40 min than that observed in rats which received mephedrone alone (pre-treatment: $F_{(2,31)}=0.49$, $p>0.05$; challenge: $F_{(1,31)}=13.40$, $p<0.001$; time: $F_{(7,217)}=12.52$, $p<0.001$; pre-treatment x challenge: $F_{(2,31)}=1.53$, $p>0.05$; pre-treatment x time: $F_{(14,217)}=2.16$, $p<0.01$; challenge x time: $F_{(7,217)}=19.34$, $p<0.001$; pre-treatment x challenge x time: $F_{(14,217)}=1.05$, $p>0.05$, Fig 2.3A). SCH 23390 enhanced the mephedrone-induced decrease in rectal temperature at 40 and 60 min post-injection by over 0.6 °C (pre-treatment: $F_{(2,30)}=9.97$, $p<0.001$; challenge: $F_{(1,30)}=19.93$, $p<0.001$; time: $F_{(7,210)}=18.08$, $p<0.001$; pre-treatment x challenge: $F_{(2,30)}=0.51$, $p>0.05$; pre-treatment x time: $F_{(14,210)}=4.07$, $p<0.001$; challenge x time: $F_{(7,210)}=31.87$, $p<0.001$; pre-treatment x challenge x time: $F_{(14,210)}=1.20$, $p>0.05$, Fig 2.3B). In

contrast, pre-treatment with BRL 44408 (Fig 2.3C) or L-741,626 (Fig 2.3D) did not alter the mephedrone-induced decrease in rectal temperature.

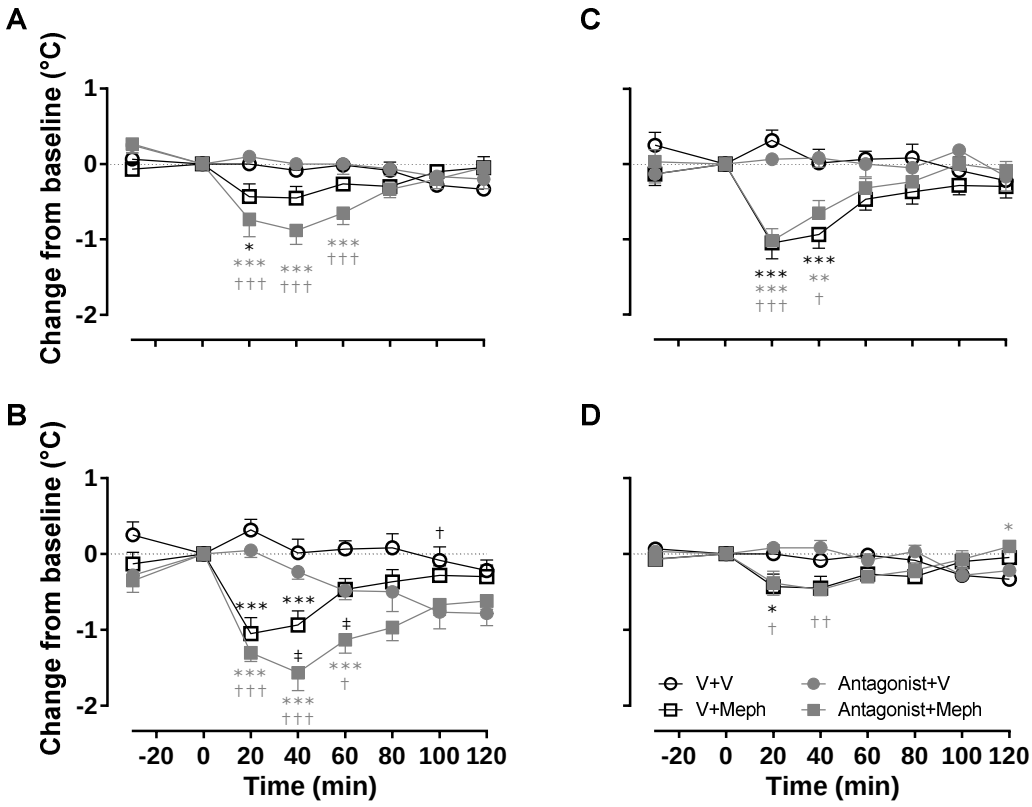


Figure 2.3 Effects of adrenoceptor or dopamine receptor antagonism on mephedrone-induced hypothermia.

Individually-housed adult male Lister hooded rats (n=6-7 per treatment group) received i.p. vehicle (1 ml kg^{-1}) or (**A**) the α_1-adrenoceptor antagonist, prazosin (0.2 mg kg^{-1}), (**B**) the dopamine D_1 receptor antagonist, SCH 23390 (2 mg kg^{-1}), (**C**) the α_{2A}-adrenoceptor antagonist, BRL 44408 (1 mg kg^{-1}), or (**D**) the dopamine D_2 receptor antagonist, L-741,626 (0.63 mg kg^{-1}) 30 min prior to i.p. injection with saline vehicle or mephedrone (10 mg kg^{-1}). Rectal temperature was recorded at pre-treatment (-30 min) and at the time of mephedrone injection (0 min) and then every 20 min for a further 2 h. Data are expressed as temperature change (°C, mean ± SEM) from baseline temperature (0 min). ***p<0.001, **p<0.01, *p<0.05 vehicle + mephedrone compared to vehicle + vehicle; ††p<0.01, †p<0.05 antagonist + vehicle compared to vehicle + vehicle; ***p<0.001, **<0.01, *p<0.05 antagonist + mephedrone compared to vehicle + vehicle; †††p<0.001, ††<0.01, †p<0.05 antagonist + mephedrone compared to antagonist + vehicle; ‡‡p<0.01, ‡p<0.05 vehicle + mephedrone compared to antagonist + mephedrone, Bonferroni multiple comparisons post-hoc following three-way repeated measures ANOVA.

2.4.2.2 Plasma catecholamine levels

Mephedrone significantly elevated the plasma noradrenaline levels 2 h post-injection in individually-housed rats ($p<0.05$, Fig 2.4A-D). This effect was abolished by pre-treatment with prazosin, BRL 44408, SCH 23390 and L-741,626. Mephedrone also appeared to increase plasma adrenaline levels but this failed to reach statistical significance in each of the individual drug studies (Fig 2.4E-H).

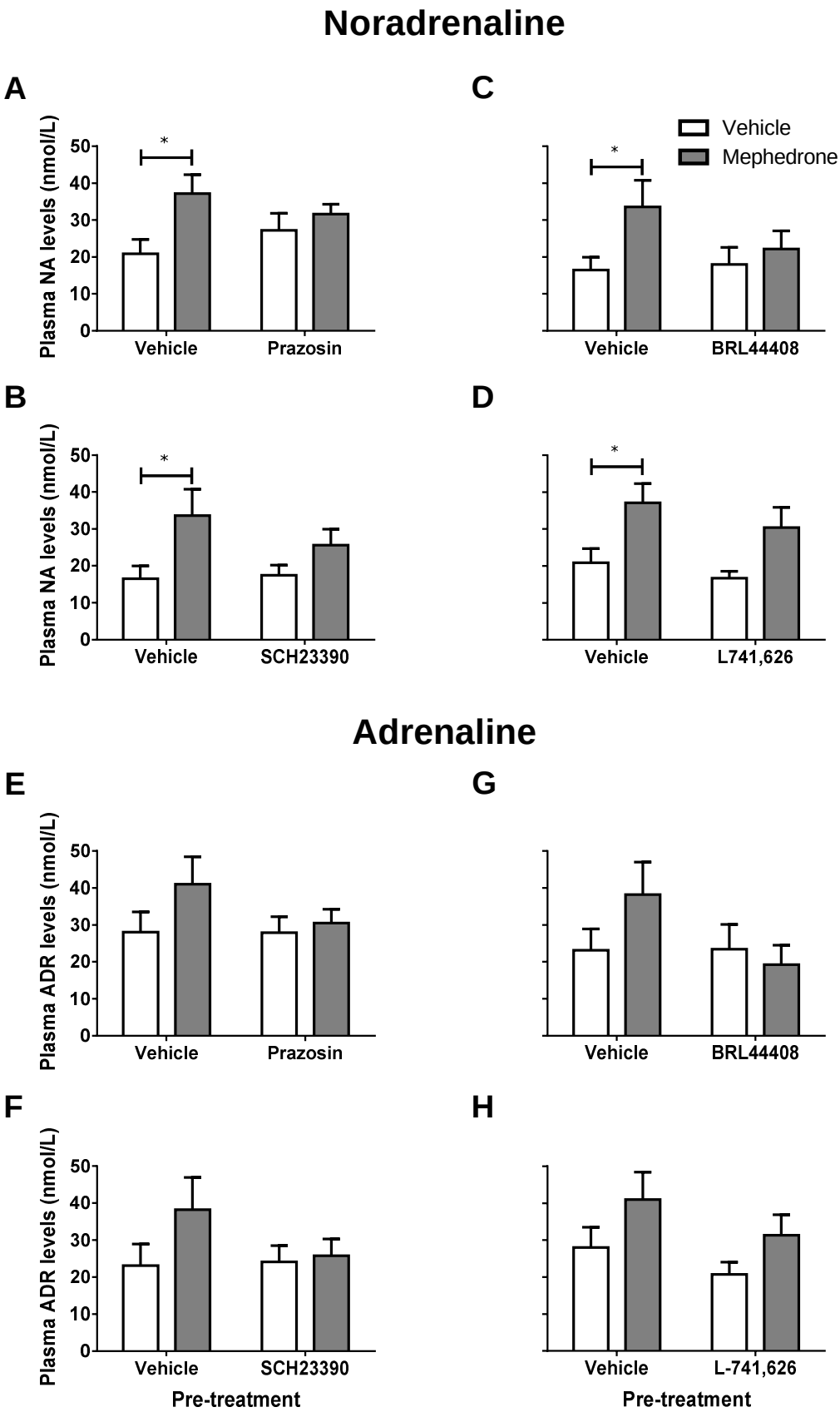

Noradrenaline
A
B
C
D
Vehicle
Mephedrone
Plasma NA levels (nmol/L)
Vehicle
Prazosin
SCH23390
BRL44408
L741,626
Adrenaline
E
F
G
H
Plasma ADR levels (nmol/L)
L-741,626
Pre-treatment

Figure 2.4 Effects of adrenoceptor or dopamine receptor antagonism on mephedrone-induced changes in plasma catecholamine levels.

Individually-housed adult male Lister hooded rats (n=6-7 per treatment group) received i.p. saline vehicle (1 ml kg^{-1}) or (**A**, **E**) the α_1-adrenoceptor antagonist, prazosin (0.2 mg kg^{-1}), (**B**, **F**) the dopamine D_1 receptor antagonist, SCH 23390 (2 mg kg^{-1}), (**C**, **F**) the α_{2A}-adrenoceptor antagonist, BRL 44408 (1 mg kg^{-1}) or (**D**, **H**) the dopamine D_1 receptor antagonist, L-741,626 (0.63 mg kg^{-1}) 30 min prior to i.p. injection with saline vehicle or mephedrone (10 mg kg^{-1}). Arteriovenous trunk blood was collected 2 h after vehicle or mephedrone injection for measurement of plasma noradrenaline (**A-D**) and adrenaline (**E-H**). *$p<0.05$ Bonferroni post-hoc following two-way ANOVA.

2.4.3 Effect of mephedrone on rectal and tail temperature, and plasma catecholamine levels in group-housed rats

There was no significant between-group difference in basal rectal or tail temperature prior to drug injection. At the time of injection (t=0 min), rectal and tail temperature was 39.2 ± 0.1 °C (mean ± SEM) and 33.0 ± 0.1 °C, respectively in vehicle, and 38.9 ± 0.1 °C and 32.9 ± 0.1 °C in mephedrone groups. Mephedrone (10 mg kg^{-1}) did not significantly alter rectal (Fig 2.5A) or tail (Fig 2.5B) temperature in group-housed rats ($p>0.05$). Plasma noradrenaline levels 2 h post-injection were 16.44 ± 3.9 nmol L^{-1} (mean ± SEM) in vehicle treated rats and 21.83 ± 4.0 nmol L^{-1} in the mephedrone treated group. Plasma adrenaline levels were 19.53 ± 6 nmol L^{-1} in vehicle treated rats and 23.4 ± 14.7 nmol L^{-1} in mephedrone treated rats. Mephedrone did not significantly alter plasma catecholamine levels 2 h post-injection in group-housed rats ($p>0.05$).

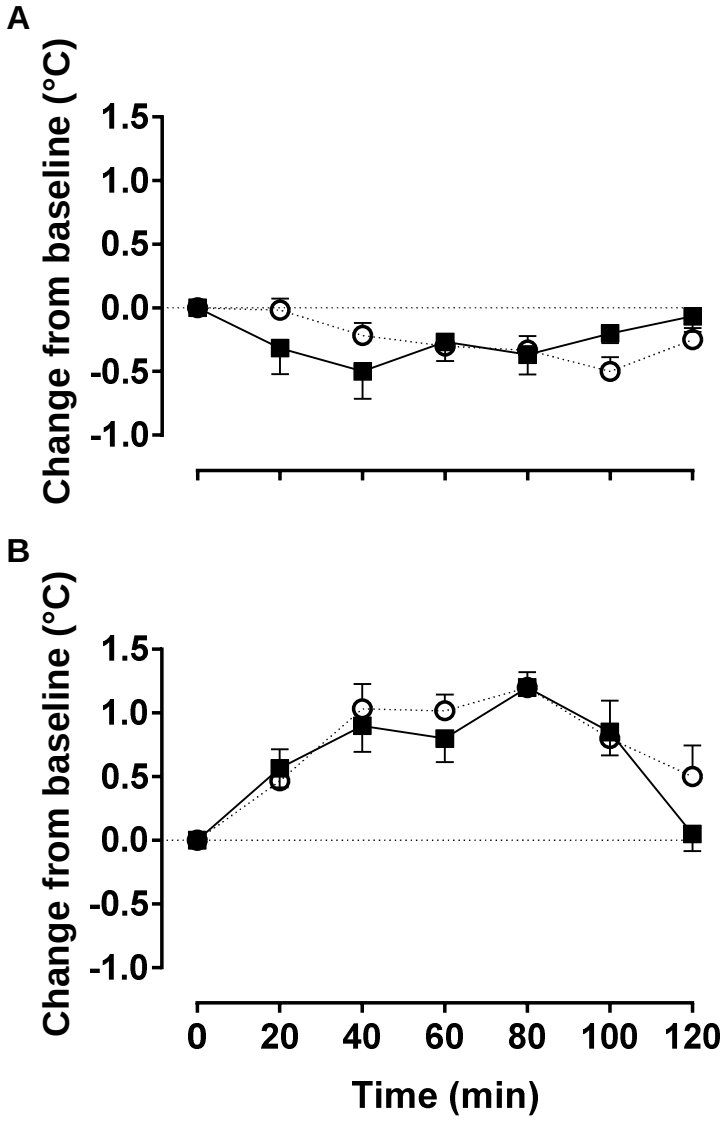


Figure 2.5 Effect of a acute mephedrone injection on rectal and tail temperature in group-housed adult male Lister hooded rats.

Group-housed adult male Lister hooded rats (n=6 per treatment group) received i.p. saline vehicle (1 ml kg^{-1}) or mephedrone (10 mg kg^{-1}) and (**A**) rectal and (**B**) tail temperatures were recorded at 20 min intervals. Data are presented as temperature change (mean ± SEM) from baseline temperature (0 min).

2.5 Discussion

In summary this study found that cathinone and methcathinone had different opposing effects on thermoregulation and post-mortem brain monoamine tissue levels to mephedrone and MDMA. Further investigations revealed that mephedrone produced a marked elevation in plasma noradrenaline levels and that the mechanism and temporal profile of mephedrone-induced hypothermia differs from that of MDMA.

MDMA can cause hyper- or hypothermia in rats depending on a number of factors, such as housing conditions or dose. In the current study, prolonged hypothermia was observed following acute MDMA administration to individually-housed rats at normal ambient temperature, as has been reported in previous studies (Malberg and Seiden 1998; Malpass et al. 1999; Daws et al. 2000; Green et al. 2005; Bexis and Docherty 2006) While mephedrone also produced hypothermia in rats, this returned to baseline levels more rapidly than that following MDMA. The speed of onset is consistent with the high brain permeability of mephedrone and its speed of onset may indicate a short plasma half-life with peak plasma levels observed 15 min after s.c. injection with the drug completely cleared after 2 h (Miller et al. 2013; Simmler et al. 2013). Recent studies have also demonstrated mephedrone-induced hypothermia following acute injection to rats at both normal (20-23 °C) and high (27 °C) ambient room temperatures (Wright et al. 2012; Miller et al. 2013) and the current study showed that group housing of rats abolished mephedrone-induced hypothermia but failed to produce hyperthermia. This is in contrast to MDMA where hyperthermia is observed at both high ambient room temperatures and with group housing (Nash et al. 1988; Colado et al. 1993; Mechan et al. 2002). At the time the current studies were conducted there were little published pharmacokinetic data for the cathinones but since the cathinones used in this study have similar molecular weights to MDMA and each other, the doses used were selected based on doses of MDMA relevant to those taken by users.

Thermoregulation is controlled by both central and peripheral mechanisms and in rats the tail is the major site of heat loss. In hot conditions, vasodilatation (with an associated increase in tail skin temperature) occurs to dissipate core body heat whereas, in cold conditions, vasoconstriction (and a decrease in tail skin temperature) serves to conserve core body heat. Since MDMA and mephedrone caused hypothermia in the current study peripheral vasoconstriction and a corresponding decrease in tail temperature would be expected in order to help normalise core body temperature. The temporal mismatch between the brief decrease in tail temperature observed in MDMA treated rats and the more sustained decrease in rectal temperature suggest disruption of normal heat conservation mechanisms by MDMA. In contrast, the sustained decreased in tail temperature which follows a brief mephedrone-induced decrease in rectal temperature appears consistent with both user reports of cold or blue fingers (Schifano et al. 2011; Winstock et al. 2011) and the affinity of mephedrone for α_1- and α_2-adrenoceptors (Simmler et al. 2012).

In marked contrast to MDMA and mephedrone, both cathinone and methcathinone caused a hyperthermic response that was maintained for 80 min following cathinone and 120 min following methcathinone administration. These findings again appear consistent with reports from users (Emerson and Cisek 1993; Kelly 2011). Previous studies have shown that cathinone produces hyperthermia in anaesthetised rats (Tariq et al. 1989) and in rabbits (Kalix 1980) while methcathinone has also been shown to produce hyperthermia in restrained rats (Rockhold et al. 1997). In the current study, hyperthermia occurred without any change in tail temperature which may suggest that these compounds are acting centrally to mediate these temperature changes without causing any change to peripheral vascular tone.

Since MDMA is a potent releaser of 5-HT and dopamine in the frontal cortex, striatum and hippocampus (Green et al. 2003), post-mortem levels of these monoamines were measured 2 h post-injection. Additionally, since the hypothalamus is essential in body temperature regulation and MDMA releases 5-HT and dopamine in this region (Benamar et al. 2008; Nisijima et al. 2012), post-mortem levels of 5-HT and dopamine were also

measured in this brain region. In addition to their differing thermoregulatory effects the cathinone derivatives and MDMA also had distinct effects on post-mortem tissue monoamine levels. MDMA decreased 5-HT and 5-HIAA in the hippocampus, hypothalamus and striatum, and the dopamine metabolites, DOPAC and HVA in the striatum. These findings are consistent with previous reports (Green et al. 2003). In contrast, cathinone increased hypothalamic and striatal 5-HT, 5-HIAA and dopamine and decreased hypothalamic DOPAC, while methcathinone increased frontal cortical, hippocampal and striatal dopamine and HVA, as well as striatal 5-HIAA. Interestingly, mephedrone had no effect on dopamine, 5-HT or their metabolites 2 h post-injection. This is surprising as microdialysis studies have shown that mephedrone is a potent releaser of both dopamine and 5-HT (Hadlock et al. 2011; Kehr et al. 2011; Baumann et al. 2012). However, the nature of *ex vivo* tissue analysis of monoamine levels means that the results are indicative of what is happening exactly when the tissue was taken and the short half-life of mephedrone would suggest that monoamine levels may have returned to normal levels when the tissue was collected, 2 h post-injection. The differential effects of the β-ketoamphetamines on thermoregulation and monoamine release matched observations by Simmler et al. (2013), where mephedrone was found to be most like MDMA as a non-selective monoamine reuptake inhibitor that stimulates dopamine and 5-HT release while cathinone and methcathinone are selective catecholamine reuptake inhibitors and releasers.

The serotonergic, dopaminergic and noradrenergic systems have all been implicated in the thermoregulatory responses to MDMA. The evidence suggests that dopamine is the most important of these neurotransmitters in evoking the temperature changes observed following MDMA administration. The dopamine D_1 receptor antagonist SCH 23390 blocks MDMA-induced hyperthermia but does not alter the hypothermic response (Mechan et al. 2002). In contrast, the dopamine D_2 receptor antagonist remoxipride has no effect on MDMA-induced hypothermia but does block the hyperthermic response (Green et al. 2005). The adrenoceptors also have an important role in disruption of thermoregulation by MDMA, where α_1-adrenoceptor antagonists (prazosin) and α_2-adrenoceptor antagonists (BRL 44408) produce a biphasic temperature response with initial hypothermia developing into hyperthermia (Bexis and Docherty 2005;

Bexis and Docherty 2008); and the β_3-adrenoceptor antagonist, SR59230A, attenuates the hyperthermic response to MDMA (Bexis and Docherty 2009).

Based on the findings of the current study it would appear that mephedrone is influencing temperature changes via different mechanisms to MDMA. Unlike MDMA, mephedrone-induced hypothermia was not influenced by pre-treatment with a D_1 dopamine receptor antagonist; instead it was enhanced and prolonged by the D_2 dopamine receptor antagonist, SCH 23390. In addition, while both α_1- and α_2-adrenoceptor antagonists cause a biphasic temperature response with MDMA administration, only the α_1-adrenoceptor antagonist, prazosin prolonged the hypothermic response following mephedrone administration. Mephedrone increased plasma noradrenaline levels in a similar manner to MDMA (Hysek et al. 2011; Hysek et al. 2012), and this effect was blocked by α_1-, α_2-adrenoceptor and D_1 dopamine receptor antagonism. Further demonstrating the differential effects of mephedrone and MDMA on body temperature changes, while group housing conditions causes hyperthermia in MDMA treated rats (Docherty and Green 2010), this failed to alter the thermogenic response to mephedrone. These differences may be due to the differing affinities that these compounds have for the monoamine transporters, with mephedrone causing greater dopamine release than MDMA.

In conclusion, the current data suggests that despite their similar structures, the cathinone derivatives produce thermoregulatory changes very different to each other and to MDMA. In addition, mephedrone-induced temperature changes do not appear to be influenced by the same manipulations of environment and/or housing conditions that affect MDMA-induced changes. Therefore care should be taken when extrapolating the effects of these and other compounds and it is likely that each novel synthetic cathinone derivative will require individual study.

Since MDMA causes hyperactivity, changes in cognition and sensorimotor gating, as well as long term neurotransmitter changes, the following

chapter will investigate the effects of chronic intermittent mephedrone on locomotion, cognition and sensorimotor gating and long term *ex vivo* monoamine levels in specific brain regions, in comparison to cathinone and MDMA. The possible development of locomotor sensitisation will also be assessed.

Chapter 3 Behavioural and neurochemical effects of repeated intermittent cathinone, mephedrone or MDMA administration

3.1 Introduction

Although the data presented in the previous study did not show any post-mortem changes to monoamine tissue levels in specific brain regions 2 h following acute mephedrone injection, other published work has indicated that mephedrone alters central monoaminergic neurotransmission (Kehr et al. 2011; Baumann et al. 2012; Martinez-Clemente et al. 2012; Motbey et al. 2012). Additionally, mephedrone causes hyperactivity, conditioned place preference and reduced social preference in rats (Kehr et al. 2011; Baumann et al. 2012; Motbey et al. 2012), improves visuo-spatial learning and memory in non-human primates (Wright et al. 2012), impairs working memory in humans (Freeman et al. 2012), and previous repeated high doses disrupt short term learning and memory in rats (Motbey et al. 2012). MDMA causes visual learning and memory deficits in rats (Morley et al. 2001; Camarasa et al. 2008; Rodsiri et al. 2011) and acute doses reduce auditory and visual PPI in rats (Kehne et al. 1996; Vollenweider et al. 1999), and repeated cathinone disrupts auditory PPI in rats (Banjaw et al. 2005).

The current study therefore examined the effects of mephedrone in behavioural paradigms regulated by monoamine function, and compared its effects to those of cathinone and MDMA in the adult rat, specifically, locomotor activity and visual recognition memory, and since the amphetamines have been reported to affect associative memory and sensorimotor gating (Cappell et al. 1972; Vollenweider et al. 1999) these behaviours were also measured. The NOD task was used to assess hippocampal related recognition memory, CER was used to investigate any potential effects on amygdala and/or hippocampal dependent associative memory and finally any changes in mesolimbic dopamine dependent PPI were assessed. These tasks are sensitive to a range of dopaminergic and serotonergic manipulations and are routinely used in our laboratory (King et al. 2004; Jones et al. 2011; Rodsiri et al. 2011; Watson et al. 2011; Woods et al. 2012; McIntosh et al. 2013) and rely on the rat's natural instinct so do not require training and are therefore ideal for investigating the short term effects of the test compounds.

In the current study a dose schedule involving injection on two consecutive days each week for three weeks was used to mimic typical patterns of weekend recreational drug use. Doses of cathinone were selected from previous studies in an attempt to produce plasma levels similar to those reported in man (Feyissa and Kelly 2008). While an MDMA dose of 10 mg kg^{-1} is higher than that required to produce plasma levels comparable to that observed in recreational users (Green et al., 2012) it was chosen to reflect the longer plasma half-life of the drug in humans compared with rats (Green et al., 2012). Although it has been reported that human users of mephedrone may on average ingest 0.5-4 g in a single session, this is generally a cumulative dose following binge-style dosing (Schifano et al. 2011; Winstock et al. 2011). Previous studies have shown that significant dopamine release in the rat nucleus accumbens following a 3 mg kg^{-1} mephedrone dose (Kehr et al., 2011) so doses of 1, 4 and 10 mg kg^{-1} of mephedrone were selected to produce pharmacological effects in the current study and to allow observation of resulting behavioural changes. Low doses of cathinone have been shown to increase locomotor activity and rearing in rats (Glennon et al. 1987; Banjaw et al. 2003; Banjaw et al. 2005; Banjaw et al. 2006). Additionally the extent of the hyperthermic response (>1 °C change in rectal temperature) evoked by cathinone in the previous chapter was taken into account when selecting doses of this compound as repeated doses may have a cumulative effect. Doses of 1 or 4 mg kg^{-1} of cathinone were therefore chosen for comparison to mephedrone. Since repeated MDMA administration can result in behavioural sensitisation (Dafters 1995; Aberg et al. 2007; Atkins et al. 2009), locomotor activity was measured at both the start and end of the study to identify any locomotor sensitisation to these compounds that may have developed across the period of cognitive testing. Rats were killed seven days after the final drug administration and the concentration of dopamine, 5-HT and their metabolites were measured in the frontal cortex, striatum and hippocampus to ascertain whether the two cathinone compounds produced any longer-term monoaminergic neurotoxicity such as previously reported following repeated administration of high MDMA doses (Green et al. 2003; Carvalho et al. 2012).

Working memory is impaired in intoxicated mephedrone users (Freeman et al. 2012) so in the current study behavioural testing was performed on the

same day as drug administration to investigate the short term effects of the test compounds in the behavioural paradigms used. Tests were also conducted in order of least to most aversive to limit the effects of the previous behavioural paradigm test while minimising animal use to comply with the 3R's of humane animal testing. Drugs were administered 30 min prior to NOD and PPI tasks for consistency with the studies presented in the previous chapter. On LMA test days, LMA recording was initiated immediately post-injection so that a time course of mephedrone-induced hyperactivity could be observed. For the CER task, rats were injected immediately after testing on the conditioning day to avoid any potential analgesic properties of these test compounds but still allowing any drug effects on memory consolidation after the trial. On the second (retention) day of CER rats were again injected after the trial so that the rat received a second injection for that week.

3.2 Aims

The aims of this study were to:

(1) compare the effects of chronic intermittent cathinone, mephedrone and MDMA on locomotor activity, visual learning and memory, associative memory and sensorimotor gating in the rat;

(2) ascertain whether this dosing schedule used resulted in the development of locomotor sensitisation to these compounds across the period of cognitive testing;

(3) identify any neurotoxic effects of these compounds seven days after the completion of chronic intermittent dosing.

3.3 Materials and Methods

3.3.1 Animals

All experiments used experimentally naïve young-adult male Lister hooded rats (170-250g; Charles River UK or University of Nottingham BSU) housed in groups of 4-5 per cage under constant environmental conditions as described in section 2.3.1. All experiments were conducted during the light phase, between 9.00 and 16.00 h. The doses of drugs used were chosen to comply with the three R's of humane animal testing. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986, and ARRIVE guidelines with approval of University of Nottingham Local Ethical Committee. The body weight of each rat was taken before the first injection to ensure no basal weight differences between groups and then monitored throughout the remainder of the experiment.

3.3.2 Drugs

(-)-cathinone-HCl was obtained from Sigma-Aldrich; (±)-mephedrone-HCl was purchased from Ascent Scientific and (±)-MDMA-HCl from Tocris Bioscience. All drugs were dissolved in 0.154 M saline and doses are quoted as the salt.

3.3.3 Effects of chronic intermittent cathinone, mephedrone or MDMA on behaviour and *ex vivo* neurochemistry

3.3.3.1 Experimental design

Rats (n=6-8 per treatment group) received i.p. injections of saline vehicle (1 ml kg^{-1}), (-)-cathinone-HCl (1 or 4 mg kg^{-1}), (±)-mephedrone-HCl (1, 4 or 10 mg kg^{-1}) or (±)-MDMA-HCl (10 mg kg^{-1}) on two consecutive days a week for three weeks (day 1, 2, 8, 9, 15 and 16 of the experiment, see Fig 3.1 for a summary of the dose schedule), with behavioural testing to

evaluate effects on LMA (day 1 and again on day 16), NOD (day two), CER (days eight and nine), and PPI (day 15). Seven days after the final dose (day 23) brain tissue was collected for neurochemical measurements.

In all experiments, rats were allocated to a treatment group using a pseudorandom design but initial body weights were checked to ensure no basal weight differences between groups. Experiments were performed as three separate studies broken into vehicle and cathinone (1 and 4 mg kg^{-1}); vehicle and mephedrone (1 and 4 mg kg^{-1}); and vehicle, mephedrone (10 mg kg^{-1}) and MDMA (10 mg kg^{-1}). All apparatus were cleaned with 20% ethanol prior to use and between animals to remove any odour cues.

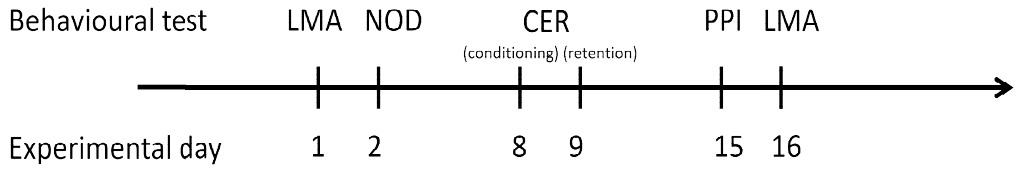


Figure 3.1 Experimental design

Young-adult male Lister hooded rats (n=6-8 per treatment group) received i.p. saline vehicle (1 ml kg^{-1}), cathinone (1 or 4 mg kg^{-1}), mephedrone (1, 4 or 10 mg kg^{-1}) or MDMA (10 mg kg^{-1}) twice weekly on consecutive days when locomotor activity (LMA, day 1 and 16), novel object discrimination (NOD, day 2), conditioned emotional response (CER, day 8 and 9) and prepulse inhibition of the acoustic startle response (PPI, day 15) were measured.

3.3.3.2 Locomotor activity

LMA was measured in a novel arena on day 1 of the experiment and reassessed in the same arena on day 16 to assess potential development of drug-induced sensitisation or tolerance. Rats were habituated to individual test arenas (as described in section 2.3.3.1) for 60 min pre-injection on each day during which LMA was recorded using a Photobeam Activity System (San Diego Instruments, CA) consisting of two layers of infra-red beams (the lower layer had eight parallel beams along its length and four parallel beams along its width, and the upper layer had eight parallel beams along its width only). Rats were then injected and immediately returned to their box where LMA was recorded for a further 60 min. Cumulative beam breaks were recorded in 5 min time bins. Activity counts were differentiated into locomotion (two consecutive adjacent lower beam breaks), fine movement (one lower beam break) and rearing (one upper beam break).

3.3.3.3 Novel object discrimination

Visual working memory is impaired in intoxicated human mephedrone users (Freeman et al. 2012) so NOD was assessed on day 2 to determine any drug-induced impairments in visual learning and memory in rats, using a protocol routinely used in our laboratory (King et al. 2004; King et al. 2009; Watson et al. 2011; McIntosh et al. 2013). Rats were returned to their individual LMA test arena 27 min following injection for an additional 3 min habituation. This was followed by 1 min in the home cage and then two consecutive 3 min object exploration trials, separated by a 2 h inter-trial interval in the home cage. In the first (familiarisation) trial rats encountered two identical objects (water filled cylindrical plastic bottles, 8 cm high and 5 cm diameter, covered in white masking tape). For the second (choice) trial one of these objects was randomly replaced with a novel object of the same size and shape but with four additional horizontal black stripes of black electrical insulation tape. The objects were placed at the front left and back right of the arenas, 5 cm from the side and 10 cm from the end wall.

Active exploration of the objects (defined as sniffing, licking, chewing or having moving vibrissae whilst directing the nose towards and ≤1 cm from the object) was recorded using stopwatches but climbing on objects in the absence of directed interest was excluded. Actual times spent exploring the objects in the choice trial were used to calculate the discrimination ratio [novel/(total choice trial object exploration)], where a discrimination ratio >0.5 indicates more time spent exploring the novel than the familiar object.

3.3.3.4 Conditioned emotional response

CER was performed on day 8 (conditioning) and day 9 (retention) of the study to measure any potential drug-induced changes in associative memory, using a previously described protocol (Jones et al. 2011; Woods et al. 2012; McIntosh et al. 2013) with some modifications. This paradigm utilised a two compartment box (25 x 25 x 27cm internal, Panlab S-Lab, Spain), with one light white-walled and one dark black-walled chamber, each with Perspex door fronts, separated by a computer operated door (8 x 8 cm). Each chamber had a grid floor connected to the shuttle box control unit, as well as a light and a centrally located speaker. On the conditioning day rats were individually placed in the light side of the apparatus and after 30 s the door opened, latency to enter the dark side (≤5 min in all cases) was recorded, and the door closed. After 30 s habituation to the dark side of the apparatus rats received a 5 s light (200 LUX) and tone (conditioned stimulus, 89 dB, 3 kHz) followed by a 1 s foot shock (unconditioned stimulus, 0.4 mA) in the last second of the conditioned stimulus. The light, tone and foot shock combination was repeated twice, at 1 min intervals. Rats were removed after the final foot shock, immediately injected and returned to the home cage. The duration of freezing (defined as absence of all movement except that required for respiration) during the 1 min periods following first and second foot shocks was recorded using separate stopwatches to assess acquisition of the association between conditioned and unconditioned stimuli.

Twenty-four hours later, rats were returned to the dark side of the apparatus and freezing duration recorded across a 5 min period (without cue or foot shock) as an index of contextual fear motivated associative learning and memory. The light and tone were then presented (without foot shock) and freezing duration recorded for a further 5 min to assess cued fear motivated associative learning and memory. Again, rats were dosed immediately after the session so that they received two injections that week.

3.3.3.5 Prepulse inhibition of acoustic startle response

PPI was measured on day 15 to assess drug-induced changes in sensorimotor gating using a previously described protocol (Jones et al. 2011; McIntosh et al. 2013). Four SR-lab startle response chambers (San Diego Instruments, CA) were used, each consisting of a clear Perspex cylinder (8.8 x 19.5 cm) mounted on a piezoelectric transducer and contained within a sound-attenuating individually ventilated chamber (39 x 38 x 58 cm). Test sessions commenced 30 min after injection and consisted of 5 min acclimatisation to background white noise (62 dB), then ten successive startle alone pulses (120 dB) followed by 50 further startle trials (ten without any pre-pulse and ten each of startle preceded by a 72 dB, 76 dB, 80 dB and 84 dB pre-pulse in a pseudorandom order and with an unpredictable inter-trial interval) and ending with five startle-alone pulses. Individual whole body startle responses were recorded every 1 ms over a 100 ms period starting from the initiation of the startle pulse using Startle Reflex Testing software (San Diego Instruments, CA) to calculate a total cumulative area under the curve (AUC) response. Results were expressed as percentage PPI from the average AUC for each trial type (using a conditional statement to eliminate any extreme values ± 2 SD from the mean which can result from movement of the rat during startle delivery) using the equation % PPI = [((pulse alone AUC - prepulse AUC)/pulse alone AUC) x 100].

3.3.3.6 Tissue collection and neurochemical detection by HPLC-ED

Rats were killed seven days after the final injection by concussion and immediate decapitation. Right striatum, frontal cortex and hippocampus were rapidly dissected on a refrigerated table (BC72: Osborne refrigeration, UK; 4 °C), weighed, flash frozen in liquid nitrogen and stored at -80 °C until analysis. HPLC-ED was performed as described in section 2.3.3.2.

3.3.4 Statistical analysis

All statistical analyses were performed using GraphPad Prism (v 6.02) or SPSS v 21 software. Experiments were performed as three separate studies broken into vehicle and cathinone (1 and 4 mg kg^{-1}); vehicle and mephedrone (1 and 4 mg kg^{-1}); and vehicle, mephedrone (10 mg kg^{-1}) and MDMA (10 mg kg^{-1}). Statistical analyses were performed against the relevant vehicle control group for each of the three individual studies. LMA time course data were analysed by two-way repeated measures ANOVA, with drug treatment and time as the between- and within-group factors, respectively. Total activity counts were also analysed by two-way repeated measures ANOVA, with drug treatment and LMA day as the between- and within-group factors. NOD familiarisation and choice trial data were analysed by two-way repeated measures ANOVA, with drug treatment and object as the between- and within-group factors. NOD total object exploration data were analysed by two-way repeated measures ANOVA, with drug treatment and NOD trial as the between- and within-group factors. For the CER data, latency to enter the dark side was analysed by one-way ANOVA. Freezing behaviour on the conditioning and retention days were analysed by two-way repeated measures ANOVA with treatment and stimuli as the as the between- and within-group factors. % PPI was analysed by two-way repeated measures ANOVA with treatment and prepulse amplitude as the between- and within-group factors. Startle response magnitude to the 120 dB prepulse alone, as well as NOD choice trial discrimination ratio and HPLC-ED data were analysed by one-way ANOVA. Bonferroni multiple comparisons post-hoc tests were conducted where appropriate. In each case, $p<0.05$ was considered significant. In

addition, one sample *t*-tests with a hypothetical mean of 0.5 were applied to NOD choice trial discrimination ratio data for each treatment group.

3.4 Results

3.4.1 Effects of chronic intermittent cathinone, mephedrone or MDMA on behaviour and *ex vivo* neurochemistry

3.4.1.1 Body weight

All rats gained weight throughout the experiment ($p<0.001$ for all groups) irrespective of treatment (Cathinone study treatment: $F_{(2,21)}=0.14$, $p>0.05$; low dose mephedrone study treatment: $F_{(2,21)}=0.70$, $p>0.05$; mephedrone and MDMA 10 mg kg^{-1} study treatment: $F_{(2,19)}=1.17$, $p>0.05$, Fig 3.2).

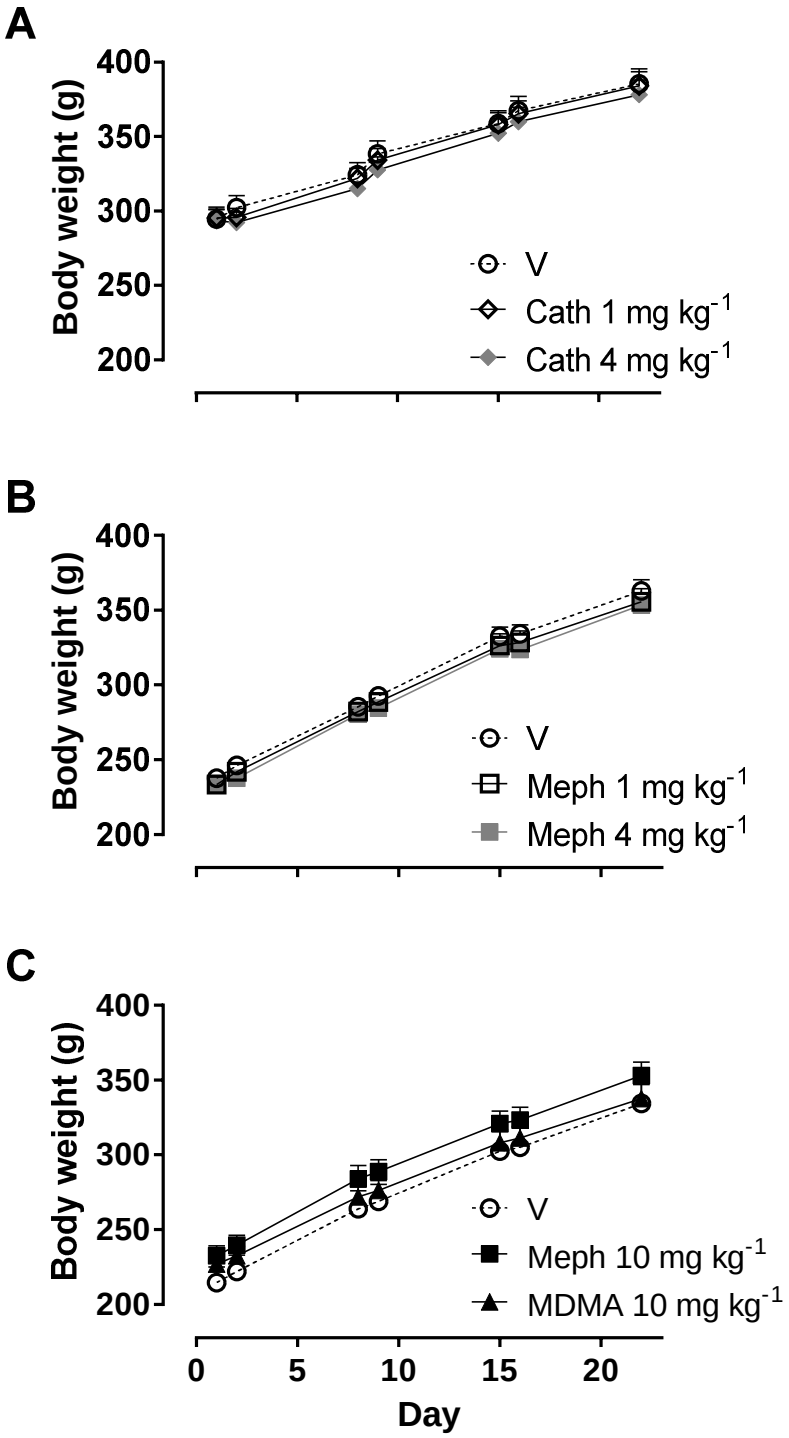


Figure 3.2 Chronic intermittent drug treatment did not affect body weight.

Adult male Lister hooded rats (n=6-8 per treatment group) displayed a normal increase in body weight (g, mean ± SEM) throughout the 4 week experiment which was unaffected by twice weekly i.p. injection on consecutive days of saline vehicle (V; 1 mg kg^{-1}), (**A**) cathinone (Cath; 1 or 4 mg kg^{-1}), (**B**) mephedrone (Meph; 1 or 4 mg kg^{-1}) or (**C**) mephedrone or MDMA (10 mg kg^{-1}).

3.4.1.2 Locomotor activity

Habituation to the activity boxes prior to injection on days 1 and 16 was confirmed by a decline in locomotion over the 60 min pre-injection period on each occasion, with no significant between-group difference in any study (Fig 3.3). On day 1 there was a marked increase in locomotion after the first injection of cathinone, mephedrone or MDMA. This effect first reached statistical significance from the relevant vehicle control group at 35 ($p<0.001$) and 15 ($p<0.05$) min post-injection for 1 and 4 mg kg^{-1} of cathinone respectively (treatment: $F_{(2,21)}=41.65$, $p<0.001$; time: $F_{(12,252)}=7.17$, $p<0.001$ treatment x time interaction: $F_{(24,252)}=7.66$, $p<0.001$, Fig 3.4A), 10 min ($p<0.001$) post-injection for 4 mg kg^{-1} mephedrone (treatment: $F_{(2,21)}=2.96$, $p=0.07$; time: $F_{(12,252)}=11.32$, $p=0.07$; treatment x time interaction: $F_{(24,252)}=1.92$, $p<0.01$, Fig 3.4B), and 10 min post-injection ($p<0.01/p<0.05$ respectively) for 10 mg kg^{-1} of mephedrone and MDMA (treatment: $F_{(2,19)}=9.91$, $p<0.001$; time: $F_{(12,228)}=9.15$, $p<0.001$; treatment x time interaction: $F_{(24,228)}=4.23$, $p<0.001$, Fig 3.4C). This elevation in locomotion was transient following mephedrone (4 and 10 mg kg^{-1}) but prolonged following cathinone (4 mg kg^{-1}) and MDMA (10 mg kg^{-1}) with no return to baseline levels by 60 min post-injection.

Similar responses to drug administration were obtained on day 16 following the sixth dose. As on day one, cathinone and MDMA caused a prolonged increase in horizontal activity (cathinone 4 mg kg^{-1}; treatment: $F_{(2,21)}=11.46$, $p<0.001$; time: $F_{(12,252)}=6.52$, $p<0.001$; treatment x time interaction: $F_{(24,252)}=7.84$, $p<0.001$, Fig 3.4D, MDMA 10 mg kg^{-1}; treatment: $F_{(2,19)}=9.91$, $p<0.001$; time: $F_{(12,228)}=8.35$, $p<0.001$; treatment x time interaction: $F_{(24,228)}=3.83$, $p<0.001$ Fig 3.4F) such that there was no return to baseline levels within the 60 min test period. Mephedrone (4 mg kg^{-1}) had a similar transient effect on locomotion on day 16 as observed on day 1 (treatment: $F_{(2,21)}=4.13$, $p<0.05$; time: $F_{(12,252)}=16.27$, $p<0.001$; treatment x time interaction: $F_{(24,252)}=3.18$, $p<0.001$, Fig 3.4E), however, of particular note the time course of hyperactivity following the 10 mg kg^{-1} dose of mephedrone was more prolonged on day 16 reaching statistical significance from vehicle from 10-

55 min post-injection instead of 10-15 min post-injection on day 1. There was no significant effect of the lowest dose of mephedrone (1 mg kg^{-1}) on locomotion on either day ($p>0.05$).

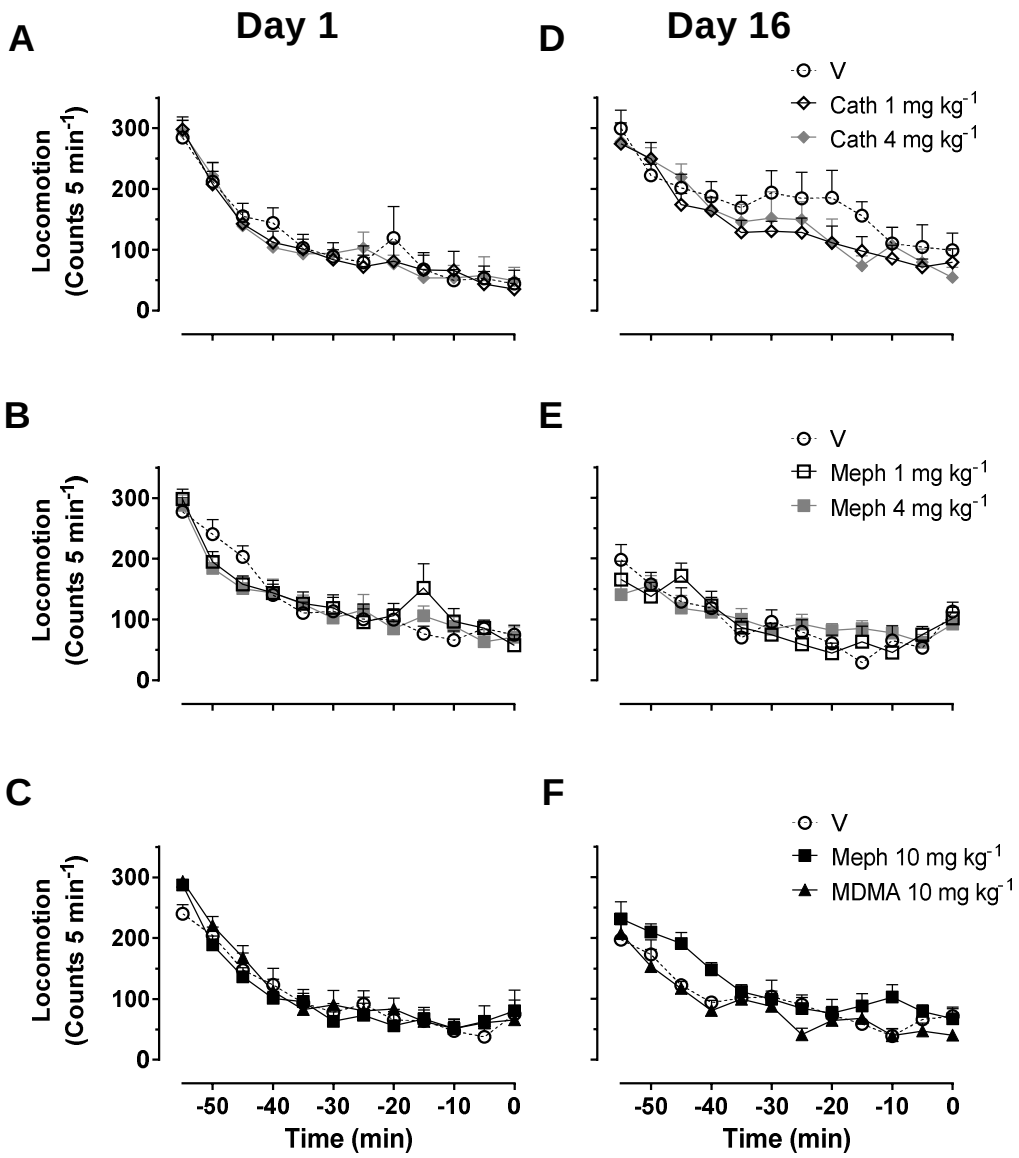


Figure 3.3 All rats displayed habituation to the activity boxes in the 60 min pre-injection period on day 1 and 16.

Adult male Lister hooded rats (n=6-8 per treatment group) displayed habituation to the activity boxes as shown by a decrease in horizontal activity counts (mean ± SEM) on days (**A-C**) one and (**D-F**) 16 of the experiment prior to the first or sixth i.p. injection of saline vehicle (V; 1 ml kg^{-1}) or (**A**, **D**) cathinone (Cath; 1 or 4 mg kg^{-1}), (**B**, **E**) mephedrone (Meph; 1 or 4 mg kg^{-1}) or (**C**, **F**) Meph or MDMA (10 mg kg^{-1}) at time=0 min. There was no significant difference in horizontal activity counts between treatment groups, two-way repeated measures ANOVA.

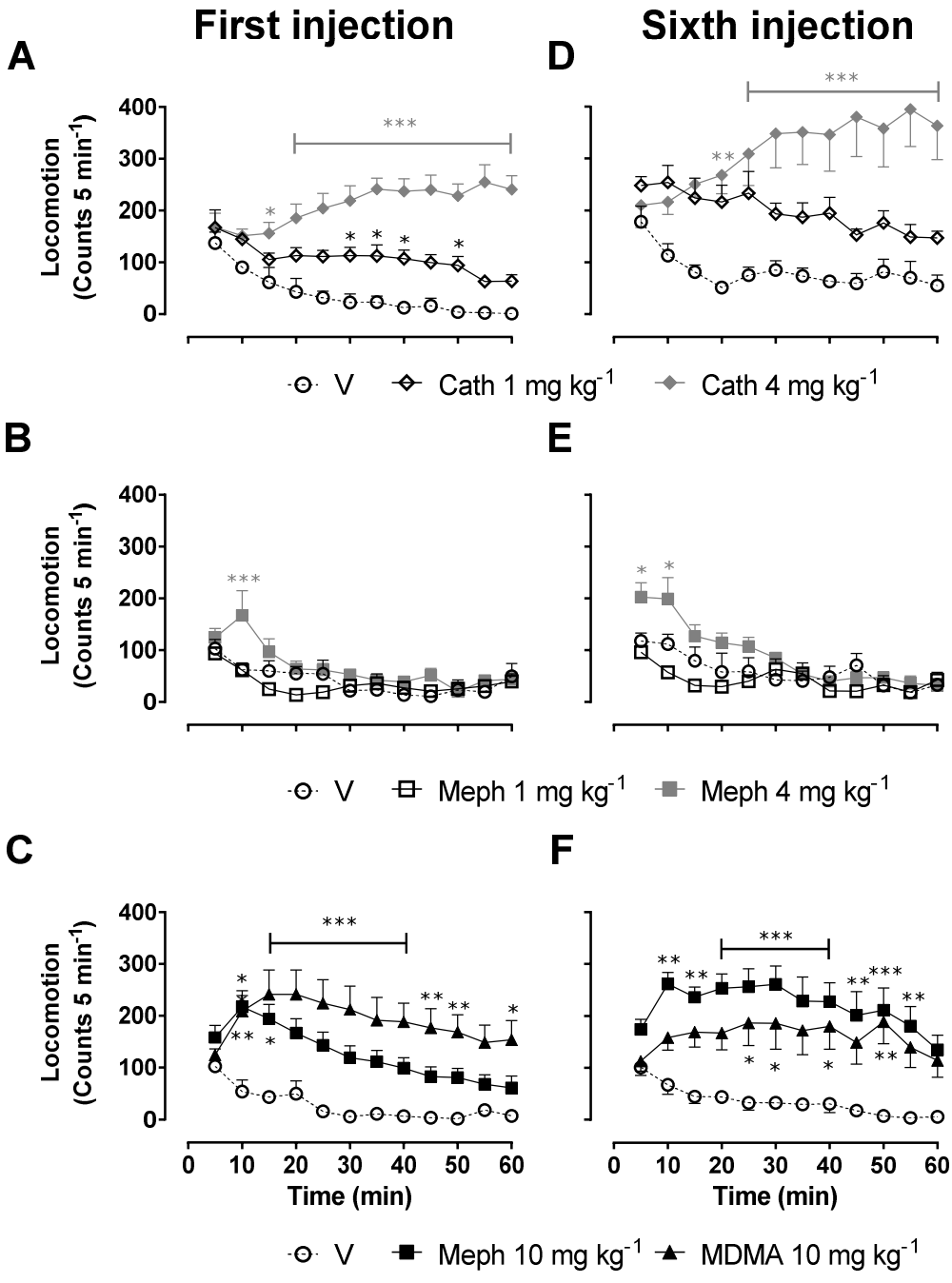


Figure 3.4 Effects of acute and chronic intermittent cathinone, mephedrone and MDMA on locomotor activity.

Adult male Lister hooded rats (n=6-8 per treatment group) displayed hyperactivity as shown by an increase in horizontal activity counts (mean ± SEM) on days (**A-C**) one and (**D-F**) 16 of the experiment following the first or sixth i.p. injection of saline vehicle (V; 1 ml kg^{-1}) or (**A**, **D**) cathinone (Cath; 1 or 4 mg kg^{-1}), (**B**, **E**) mephedrone (Meph; 1 or 4 mg kg^{-1}) or (**C**, **F**) Meph or MDMA (10 mg kg^{-1}) at time=0 min. Experiments were performed as three separate studies broken into vehicle and cathinone (1 and 4 mg kg^{-1}); vehicle and mephedrone (1 and 4 mg kg^{-1}); and vehicle, mephedrone (10 mg kg^{-1}) and MDMA (10 mg kg^{-1}). Statistical

analyses were performed for each individual study against its own vehicle control group. *p<0.05, **p<0.01, ***p<0.001 compared to saline vehicle, Bonferroni post-hoc following two-way repeated measures ANOVA.

Further analysis of the cumulative horizontal activity counts over the entire 60 min post-injection recording period showed that cathinone (4 mg kg^{-1}) and MDMA (10 mg kg^{-1}) both produced significant increases on day 1 ($p<0.001$ in each case, Table 3.1), but on day 16 the effects of cathinone and mephedrone were both exacerbated such that previously sub-threshold doses of 1 mg kg^{-1} cathinone and 10 mg kg^{-1} mephedrone produced a significant elevation in total horizontal locomotion counts on day 16 ($p<0.05$ and $p<0.001$ versus relevant vehicle control, respectively). Furthermore, the total ambulation counts observed following the final injection of cathinone (1 and 4 mg kg^{-1}) and mephedrone (10 mg kg^{-1}) were significantly higher than those observed in the same animals in response to the first injection ($p<0.05$-$p<0.01$).

Table 3.1 Effects of acute and chronic intermittent cathinone, mephedrone and MDMA on total horizontal ambulation counts following injection with cathinone, mephedrone or MDMA.

Treatment	First injection	Sixth injection
Vehicle	488 ± 80	1084 ± 186
Cath 1	1329 ± 120	2457 ± 254*†
Cath 4	2573 ± 242***	3851 ± 634***††
Vehicle	570 ± 113	823 ± 168
Meph 1	480 ± 103	609 ± 73
Meph 4	873 ± 139	1181 ± 166
Vehicle	395 ± 53	488 ± 73
Meph 10	1583 ± 220	2694 ± 347***††
MDMA 10	2347 ± 418***	1964 ± 416*

Cumulative horizontal activity counts (mean ± SEM) in a 60 min period following the first and sixth injections of a chronic intermittent dosing schedule in adult male Lister hooded rats (n=6-8 per treatment group). Experiments were performed as three separate studies broken into i.p. saline vehicle (V, 1 ml kg^{-1}) and cathinone (1 and 4 mg kg^{-1}); V and mephedrone (1 and 4 mg kg^{-1}); and V, mephedrone (10 mg kg^{-1}) and MDMA (10 mg kg^{-1}) on two consecutive days a week for three weeks. Statistical analyses were performed for each individual study against its own vehicle control group. *$p<0.05$, ***$p<0.001$ compared to saline vehicle following on the same day; †$p<0.05$, ††$p<0.01$ compared to the same treatment after the first injection, Bonferroni post-hoc following two-way repeated measures ANOVA.

The total cumulative number of rears during the 60 min post-injection period was increased by the first cathinone injection only (treatment: $F_{(2,21)}=8.47$, $p<0.01$), although this response did not differ between the first and sixth injections (injection number: $F_{(1,21)}=0.09$, $p>0.05$; treatment x injection number interaction: $F_{(2,21)}=0.73$, $p>0.05$, Table 3.2). In a similar pattern to horizontal locomotion, 10 mg kg^{-1} mephedrone increased the total number of rears following the sixth injection compared to the first ($F_{(1,19)}=7.60$, $p<0.01$), but MDMA had no significant effect on rearing behaviour on either test day.

The total cumulative fine movement counts during the 60 min post-injection period were also increased by cathinone ($F_{(2,21)}=118.6$, $p<0.001$, Table 3.2). This effect reached significance on both day 1 and 16 and although the absolute counts were significantly higher on day 16 compared to day 1 ($F_{(1,21)}=55.9$, $p<0.001$) the vehicle group in this experiment also displayed increased fine movement counts after the sixth injection compared to the first. Mephedrone and MDMA (10 mg kg^{-1}) increased fine movement counts after the first and sixth injections ($F_{(2,19)}=28.21$, $p<0.001$).

Table 3.2 Effects of acute and chronic intermittent cathinone, mephedrone and MDMA on total rearing and fine movement counts.

	Rearing counts		Fine movement counts	
Treatment	First injection	Sixth injection	First injection	Sixth injection
Vehicle	34 ± 8	89 ± 19	218 ± 28	455 ± 30†††
Cath 1	311 ± 127**	243 ± 40	540 ± 16***	673 ± 37***†
Cath 4	65 ± 11	121 ± 43	704 ± 33***	880 ± 31***††
Vehicle	85 ± 20	76 ± 12	308 ± 40	359 ± 50
Meph 1	108 ± 70	78 ± 18	268 ± 51	360 ± 45
Meph 4	101 ± 42	158 ± 53	373 ± 29	488 ± 37
Vehicle	33 ± 8	53 ± 18	233 ± 32	313 ± 22
Meph 10	135 ± 103	312 ± 101††	565 ± 50***	709 ± 32***
MDMA 10	23 ± 8	90 ± 34	557 ± 64***	609 ± 58***

Cumulative rearing and fine movement counts (mean ± SEM) in a 60 min period following the first and sixth injections of a chronic intermittent dosing schedule in adult male Lister hooded rats (n=6-8 per treatment group). Experiments were performed as three separate studies broken into i.p. saline vehicle (V, 1 ml kg^{-1}) and cathinone (1 and 4 mg kg^{-1}); V and mephedrone (1 and 4 mg kg^{-1}); and V, mephedrone (10 mg kg^{-1}) and MDMA (10 mg kg^{-1}) on two consecutive days a week for three weeks. Statistical analyses were performed for each individual study against its own vehicle control group. **p<0.01, ***p<0.001 compared to saline vehicle following the same number of injections, †p<0.05, ††p<0.01, †††p<0.001 compared to the first injection of the same treatment, Bonferroni post-hoc following two-way repeated measures ANOVA.

3.4.1.3 Novel object discrimination

When NOD was assessed following the second injection none of the treatment groups exhibited any spatial preference for either identical object during the familiarisation trial ($p>0.05$, Fig 3.5A). However, it is important to note that there was a significant drug-induced decrease in total levels of object exploration during this first trial following all treatments, except for the 1 mg kg^{-1} doses of cathinone and mephedrone (Fig 3.5A).

During the choice trial 2 h later, all three groups of vehicle treated rats successfully discriminated the novel from the familiar object during the choice trial, as expected, but this was impaired by all three drugs ($p<0.001$, Fig 3.5B). This redistribution of object exploration occurred without any difference in total choice trial exploration levels, with the exception of 10 mg kg^{-1} mephedrone, which produced a significant increase in exploration in the choice trial ($p<0.01$). In the case of high dose mephedrone and MDMA (10 mg kg^{-1}) the impaired discrimination translated into a significant decrease in the choice trial discrimination ratio ($F_{(2,19)}=10.11$, $p<0.001$, Fig 3.5C) which was not observed with the other treatments. Analysis of the discrimination ratio data by one sample *t*-test, against a hypothetical chance mean of 0.5, showed a similar pattern to two-way repeated measures ANOVA of novel versus familiar data, namely performance above chance levels in vehicle, but not cathinone, mephedrone or MDMA treated rats.

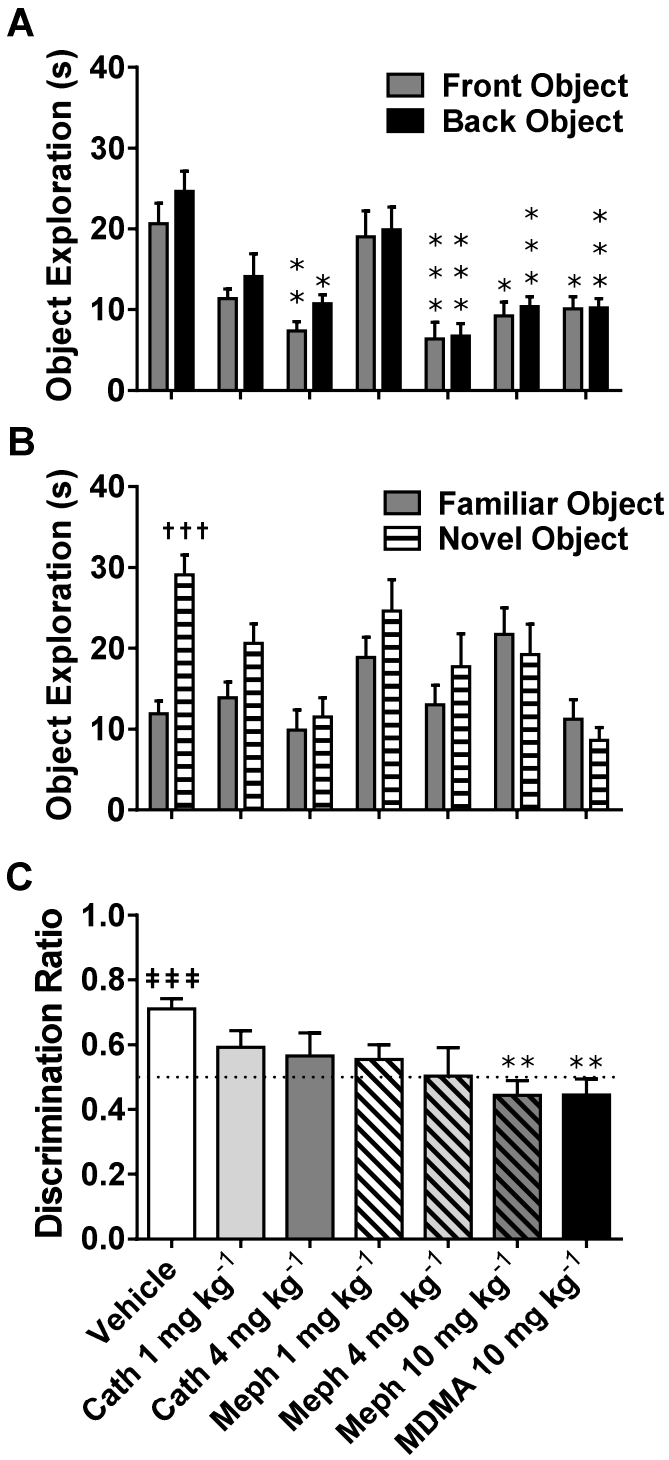


Figure 3.5 Cathinone, mephedrone and MDMA impaired novel object discrimination following the second injection in a chronic intermittent dosing regime.

Young adult male Lister hooded rats (n=6-8 per treatment group) received i.p. saline vehicle (1 ml kg^{-1}), cathinone (Cath; 1 or 4 mg kg^{-1}), mephedrone (Meph; 1, 4 or 10 mg kg^{-1}) or MDMA (10 mg kg^{-1}) on two consecutive days a week for three weeks with assessed NOD 30 min after the second injection (mean ± SEM).

Duration of object exploration (s) in the (**A**) familiarisation and (**B**) choice trials of NOD which were separated by a 2 h inter-trial interval, and the (**C**) choice trial discrimination ratio (time exploring novel object/total choice trial object exploration) were measured after the second injection. Experiments were performed as three separate studies each with their own vehicle control group and vehicle data are pooled for clarity of presentation, but statistical analyses were performed for each individual study against its own vehicle control group. **p<0.01, ***p<0.001 compared to saline vehicle, †p<0.05, ††p<0.01 compared to the familiar object in the same treatment group, Bonferroni post-hoc following two-way repeated measures ANOVA. ‡‡‡p<0.001 compared to hypothetical mean of 0.5, one sample t-test.

3.4.1.4 Conditioned emotional response

When CER conditioning was performed on day 8 of the study (7 days after the previous injection) there was no significant difference between treatment groups in the latency to cross to the dark side of the box ($p>0.05$) and all rats crossed into the dark side of the CER box within 5 min. Irrespective of treatment history, all groups exhibited comparable levels of freezing after foot shock administration (Cath 1 and 4 mg kg^{-1}: $F_{(2,20)}=0.15$, $p>0.05$; Meph 1 and 4 mg kg^{-1}: $F_{(2,21)}=0.26$, $p>0.05$; Meph and MDMA 10 mg kg^{-1}: $F_{(2,19)}=1.11$, $p>0.05$, Fig 3.6A) and all groups successfully acquired the CER, as demonstrated by a significant increase in freezing after the second foot shock compared to the first (Cath 1 and 4 mg kg^{-1}: $F_{(1,20)}=9.03$, $p<0.01$, Meph 1 and 4 mg kg^{-1}: $F_{(1,21)}=34.84$, $p<0.001$, Meph and MDMA 10 mg kg^{-1}: $F_{(1,19)}=33.24$, $p<0.001$). Drug treatments were administered immediately after the CER this trial (third injection) to prevent any drug effect on nociception or anxiety which could non-specifically alter conditioning and confound interpretation of any effect on learning and memory in this paradigm.

During the retention trial, 24 h after the third injection, rats exhibited freezing behaviour when returned to the environmental context where foot shocks had been delivered, again confirming successful acquisition of the CER. This contextual conditioning was impaired only by the highest dose of mephedrone (10 mg kg^{-1}) which significantly reduced freezing compared to that in vehicle control such that there was a main effect of treatment ($F_{(2,19)}=4.31$, $p<0.05$, Fig 3.6B). Subsequent presentation of the light and tone cue (in the absence of any further foot shock) significantly increased freezing duration for all mephedrone and MDMA groups compared to the context alone (main effects of Meph 1 and 4 mg kg^{-1}: $F_{(1,21)}=47.77$, $p<0.001$; Meph and MDMA 10 mg kg^{-1}: $F_{(1,19)}=5.11$, $p<0.01$ $p<0.001$) but there were no significant differences between any individual treatment group and vehicle control.

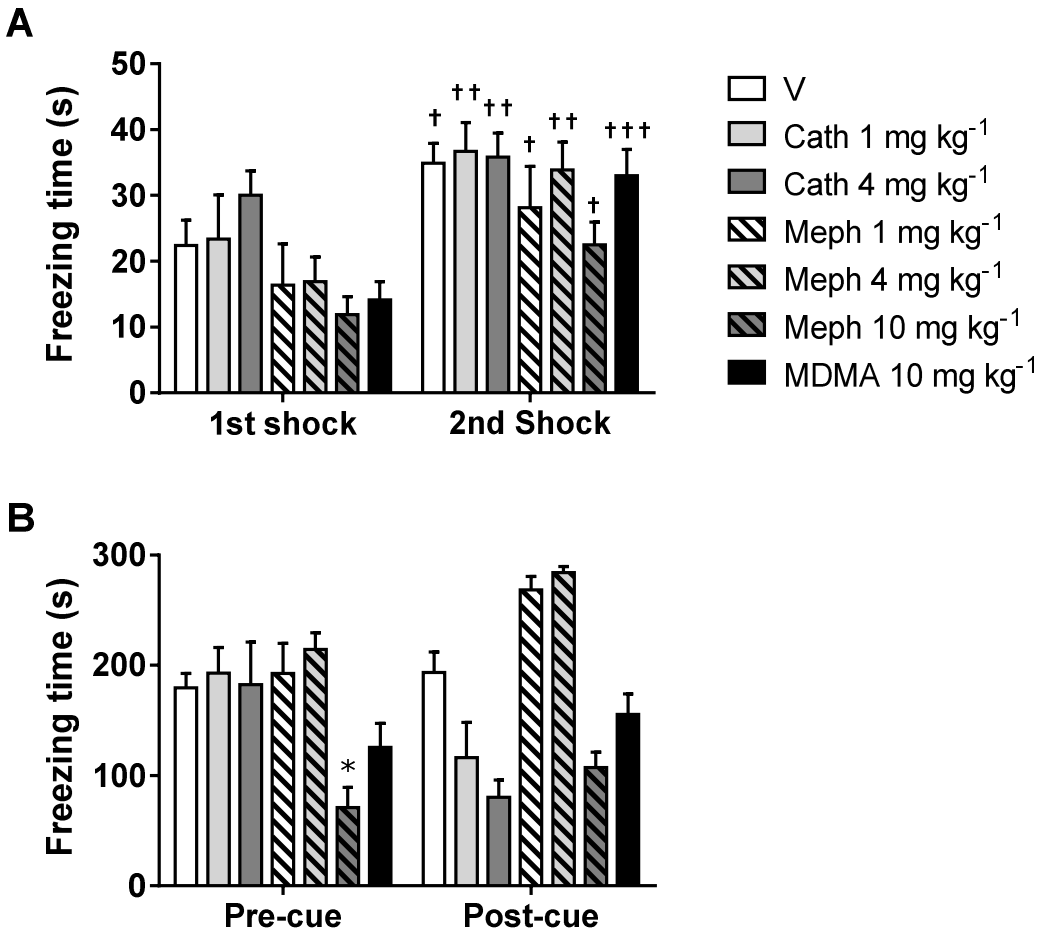


Figure 3.6 Mephedrone, but not cathinone or MDMA, impaired contextual but not cued CER.

Young adult male Lister hooded rats (n=6-8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}), cathinone (Cath, 1 or 4 mg kg^{-1}), mephedrone (Meph, 1, 4 or 10 mg kg^{-1}) or MDMA (10 mg kg^{-1}) twice weekly on consecutive days for three weeks. Duration (s, mean ± SEM) of freezing behaviour during (**A**) conditioning and (**B**) retention trials of the CER task were measured on days 8 and 9 of the experiment. Injections were administered immediately after each trial to prevent any drug effect on nociception or anxiety which could non-specifically alter conditioning and confound interpretation of any effect on learning and memory in the paradigm, such that the conditioning trial was conducted 7 days after the the second injection and the requisition trial was performed 24 h after the third injection. Experiments were performed as three separate studies each with their own vehicle control group and vehicle data are pooled for clarity of presentation, but statistical analyses were performed for each individual study against its own vehicle control group. *$p<0.01$ compared to saline vehicle, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared to freezing after the first shock, Bonferroni post-hoc following two-way repeated measures ANOVA.

3.4.1.5 Prepulse inhibition of the acoustic startle response

When PPI was assessed on day 15 of the experiment following the fifth injection, all treatment groups exhibited the normal attenuation of startle by exposure to increasing pre-pulse amplitude (Cath 1 and 4 mg kg^{-1}: $F_{(2,42)}$=38.22, p<0.001; Meph 1 and 4 mg kg^{-1}: $F_{(2,42)}$=50.21, p<0.001; Meph and MDMA 10 mg kg^{-1}: $F_{(2,38)}$=37.18, p<0.001, Fig 3.7). There were no between-group differences in % PPI at any pre-pulse amplitude (Cath 1 and 4 mg kg^{-1}: $F_{(2,21)}$=0.48, p>0.05; Meph 1 and 4 mg kg^{-1}: $F_{(2,21)}$=0.55, p>0.05; Meph and MDMA 10 mg kg^{-1}: $F_{(2,19)}$=1.45, p>0.05) and no treatment effects on either basal reactivity to the startle pulse or habituation to the startle pulse across the trial test session.

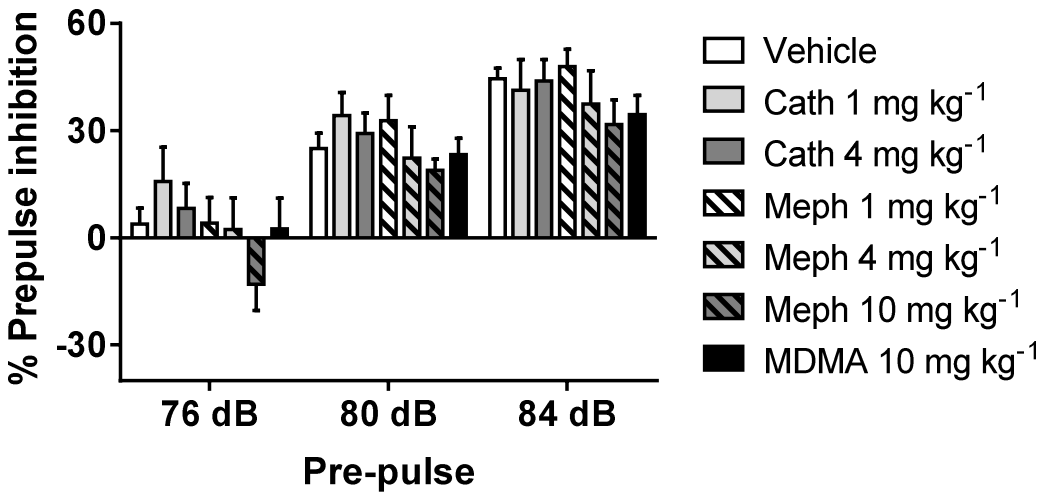


Figure 3.7 Cathinone, mephedrone and MDMA did not affect prepulse inhibition of the acoustic startle response.

Adult male Lister hooded rats (n=6-8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}), cathinone (Cath, 1 or 4 mg kg^{-1}), mephedrone (Meph, 1, 4 or 10 mg kg^{-1}) or MDMA (10 mg kg^{-1}) twice weekly on consecutive days for three weeks. Prepulse inhibition was measured on day 15 of the experiment 30 min after the fifth injection. Experiments were performed as three separate studies each with their own vehicle control group and vehicle data are pooled for clarity of presentation, but statistical analyses were performed for each individual study against its own vehicle control group.

3.4.1.6 Neurochemistry

No dose of any compound had any significant long-term effect on tissue concentrations of dopamine, 5-HT, or their metabolites in the striatum or frontal cortex seven days after the last of six chronic intermittent injections. Dopamine and 5-HT levels in the hippocampus were also unaffected (Fig 3.8A,B) but the concentration of DOPAC in this region was significantly increased following mephedrone (4 mg kg^{-1}; $F_{(2,19)}=7.37$, $p<0.01$, Fig 3.8D) and, in contrast, significantly decreased following 10 mg kg^{-1} mephedrone and MDMA ($p<0.05$; $F_{(2,19)}=33.48$, $p<0.001$ Fig 3.8D).

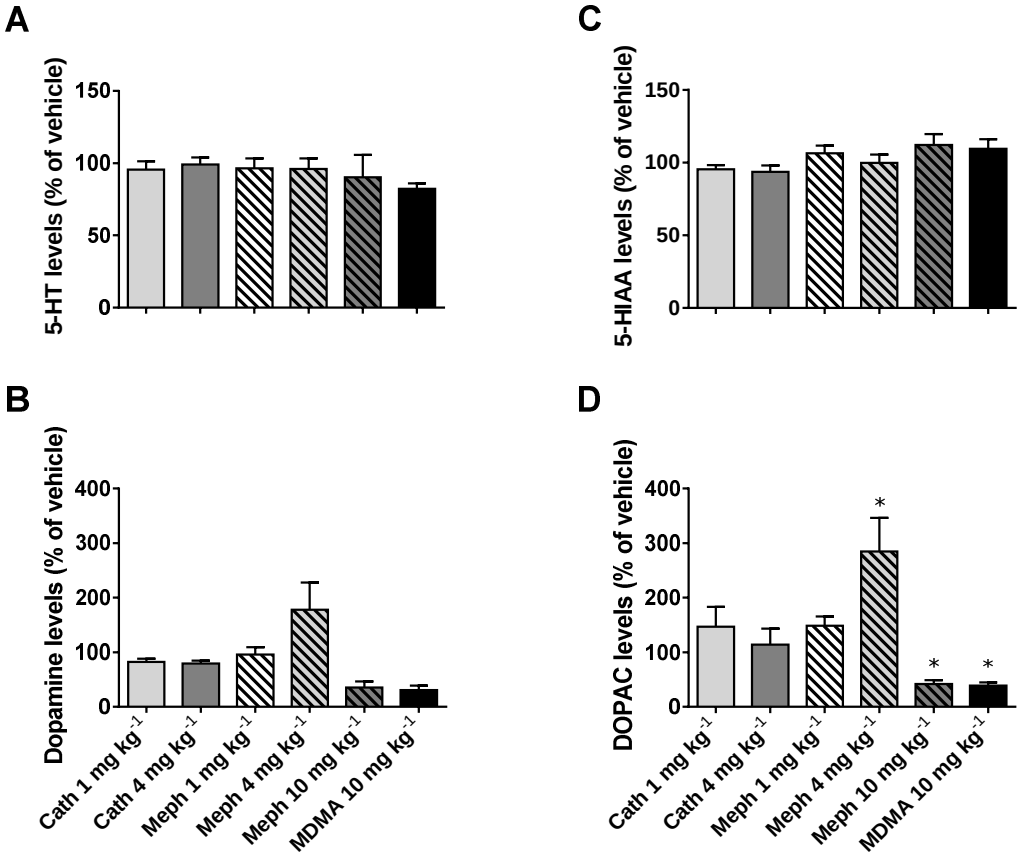


Figure 3.8. Effect of chronic intermittent cathinone, mephedrone or MDMA on hippocampal monoamine levels seven days after the last of six injections.

Adult male Lister hooded rats (n=6-8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}), cathinone (Cath, 1 or 4 mg kg^{-1}), mephedrone (Meph, 1 or 4 mg kg^{-1}) or meph or MDMA (10 mg kg^{-1}) on two consecutive days a week for three weeks. The hippocampus was dissected seven days after the final injection and analysed for (**A**) 5-HT, (**B**) dopamine, (**C**) 5-HIAA and (**D**) DOPAC levels by HPLC-ED. Experiments were performed as three separate studies each with their own vehicle control group and vehicle data are pooled for clarity of presentation, but statistical analyses were performed for each individual study against its own vehicle control group. Data are presented as a percentage of respective vehicle but statistical analysis were performed on the raw values (pmol mg^{-1} wet weight tissue, mean ± SEM). *$p<0.05$ Bonferroni post-hoc following one-way ANOVA.

3.5 Discussion

The main findings of this study were that cathinone, mephedrone and MDMA caused hyperactivity in rats following the first injection of a chronic intermittent dosing schedule, with evidence for sensitisation to cathinone- and mephedrone-induced hyperactivity by the sixth injection 16 days later. In addition, the three compounds all impaired performance in a NOD test of visual recognition memory and the highest dose of mephedrone also reduced context (but not cue) mediated freezing in the CER task. In addition, mephedrone and MDMA both altered post-mortem hippocampal DOPAC levels seven days after the final injection, but had no significant effect on either dopamine or 5-HT in the hippocampus, frontal cortex or striatum.

In the current study, cathinone, mephedrone and MDMA all produced significant ambulatory hyperactivity when administered to rats. In the case of cathinone and mephedrone (where different doses were examined) these effects appeared to be dose-dependent. Dopaminergic neuronal activity plays a key role in locomotion and dopamine release has been reported in the nucleus accumbens of rats administered cathinone (Kalix 1982), mephedrone (Kehr et al. 2011; Baumann et al. 2012) and MDMA (O'Shea et al. 2005) which may contribute to the observed hyperactivity. The hyperactivity induced by cathinone was greater in both magnitude and duration than that caused by mephedrone at the same dose. Although both cathinone (3.2 mg kg^{-1}) and mephedrone (3 mg kg^{-1}) appear to increase dopamine efflux (measured by microdialysis in the nucleus accumbens) to a peak of approximately 550% of baseline values (Pehek et al. 1990; Kehr et al. 2011) this effect is sustained in cathinone treated rats but more transient following mephedrone. Therefore, both the transient nature of mephedrone-induced hyperactivity and prolonged responses to cathinone and MDMA in the current study are consistent with previous findings by other groups (Callaway et al. 1990; Pehek et al. 1990; Bull et al. 2003; Clemens et al. 2007; Kehr et al. 2011; Kelly 2011). Interestingly, the duration of hyperactivity seen here also matches the time course of the change in body temperature observed in Chapter 2, and the transient effects of mephedrone on both LMA and temperature appear consistent

with reports from recreational users that its effects are short-lasting (Dargan et al. 2011; Winstock et al. 2011).

Behavioural sensitisation is a well established phenomenon which is seen after repeated doses of amphetamines (e.g. 5 mg kg^{-1} twice daily for five days; Robinson and Becker, 1986) and considered to result from enhanced drug-induced dopamine and glutamate release (Robinson and Becker 1982; Beutler et al. 2011), as well as increase dopamine D_1 receptor sensitivity, particularly in the nucleus accumbens and striatum (see Kalivas et al., 1983). Sensitisation to MDMA has been reported following several different dosing schedules, including 5 mg kg^{-1} once daily for seven days (Aberg et al. 2007). However behavioural sensitisation is both dose-level and dose-interval related and despite the fact that MDMA sensitisation can be induced by more intense dosing schedules, the intention in the current study was to mimic weekend recreational use, so MDMA-sensitisation was not observed in the current study. Following the sixth dose of cathinone (4 mg kg^{-1}) and mephedrone (10 mg kg^{-1}) there was a greater locomotor response than that observed following the first dose. This is consistent with reported locomotor sensitisation to cathinone after repeated daily dosing (Banjaw et al. 2005) and indicates that such sensitisation can still occur even when drug administration is intermittent. Although mephedrone-induced hyperactivity has been observed previously following single injection, repeated dosing on the same day and repeated injections over five consecutive days (Kehr et al. 2011; Baumann et al. 2012; Lisek et al. 2012; Motbey et al. 2012; Wright et al. 2012) this is the first study to show sensitisation to mephedrone following intermittent dosing.

Previous studies have indicated that MDMA administration can impair working memory and sensorimotor gating in rats (Vollenweider et al. 1999; Piper and Meyer 2004; Rodsiri et al. 2011) as well as working memory (Bolla et al. 1998; Parrott et al. 1998) and associative learning (Montgomery et al. 2005) in current and abstinent human users. In contrast, mephedrone has recently been shown to improve visuo-spatial memory and learning in non-human primates (Wright et al. 2012) but impair working memory in humans (Freeman et al. 2012), although there is no information on its cognitive effects in rodents. In the current study,

rats that had received two previous treatments with any dose of cathinone, mephedrone or MDMA (at 24 h and 30 min prior to testing) were unable to distinguish between the novel and the familiar object during the choice trial of NOD. However, during the familiarisation trial, the higher doses (4 and 10 mg kg^{-1}) of mephedrone, cathinone (4 mg kg^{-1}) and MDMA decreased total levels of object directed exploration, making it difficult to attribute the absence of choice trial discrimination at these doses to specific memory impairment, but may be due to attention deficits instead.

Neither cathinone nor MDMA influenced any aspect of associative memory in the CER test. The highest dose of mephedrone significantly reduced freezing on re-exposure to the context in which the shock was received, but did not alter freezing in response to re-presentation of the light and tone cue, suggesting that mephedrone may specifically attenuate hippocampal-dependent contextual association but not hippocampal and amygdala-dependent cued association (Phillips and Ledoux 1992; Fanselow 2000). CER acquisition was conducted seven days after the second dose and CER retention 24 h after the third dose of mephedrone. Given that the LMA response to the first and sixth mephedrone injections was significantly different from vehicle only until 15 and 55 min post-injection respectively, it is extremely unlikely that reduced freezing to context during the CER retention trial was caused by any direct locomotor effects of mephedrone injection 24 h earlier.

None of the drugs altered PPI of the acoustic startle response (sensorimotor gating) assessed 30 min after the fifth injection in this study, although importantly all vehicle treated controls showed the expected increase in PPI with increasing pre-pulse amplitude consistent with previous studies using the same equipment and protocol (Jones et al. 2011). The lack of cathinone effects herein contrast with the findings of Banjaw et al. (2005) but the dose schedule required to produce cathinone-induced deficits in PPI (2 mg kg^{-1} daily for 10 days) differed markedly from the current study. The mean initial and final startle responses to the 120 dB pulse alone and the habituation to the startle pulse across the test period were not significantly different across treatment groups, indicating

that none of the compounds exerted any additional effects which could confound interpretation of the PPI data.

Taken together, the current findings in adult male Lister hooded rats do not appear to support profound effects of the current chronic intermittent mephedrone or cathinone dosing regimen on visual or associative learning and memory or pre-attentional processing, at least in the paradigms tested. The different time points for behavioural tests resulted in animals receiving different cumulative doses before each test employed. However, since all compounds elevated LMA following the first and sixth injections, and sensitisation to cathinone and mephedrone was apparent following the sixth dose, it is unlikely that the lack of effect on PPI or CER was due to development of tolerance to any of the drugs.

Acute MDMA administration to the rat causes 5-HT release and inhibits 5-HT re-uptake, leading to decreased tissue 5-HT content (Green et al., 2003). While repeated MDMA administration induces additional neurotoxic loss of 5-HT in forebrain regions (Green et al. 2003), the extent of this neurotoxicity is dependent both on dose and frequency of administration (O'Shea et al. 1998). High dose methamphetamine also results in neurotoxic damage to dopamine and 5-HT nerve endings in the brain (Hotchkiss and Gibb 1980; Armstrong and Noguchi 2004). This study therefore examined the concentration of 5-HT and dopamine in brain regions known to be sensitive to acute and neurotoxic effects of cathinone, mephedrone or MDMA seven days following the last of six intermittent doses. There was no significant effect of any treatment on the concentration of either 5-HT or dopamine in the striatum, frontal cortex or hippocampus seven days after the sixth injection. However, mephedrone (4 mg kg^{-1}) significantly increased hippocampal DOPAC while the higher dose (10 mg kg^{-1}) caused a significant decrease. It is currently unclear what mechanism might account for this biphasic change in tissue DOPAC.

It has previously been reported that MDMA causes depletion of brain 5-HT seven days after administration (Green et al., 2003). The lack of effect of MDMA on tissue 5-HT levels seven days post-injection in the current study

may be because tissue monoamine levels are only indicative of neurotransmitter levels at a single time point of a dynamic situation involving changes in neurotransmitter release, synthesis, metabolism and elimination. Additionally, hypothermia is neuroprotective against MDMA-induced monoamine neurotoxicity (Malberg and Seiden 1998; Mueller et al. 2013). Since hypothermia was observed following acute MDMA administration in the previous chapter it is possible that this is contributing to the lack of effect of MDMA on tissue 5-HT levels seven days post-injection. However, since rectal temperature was not measured in this study this cannot be verified.

While mephedrone has recently been reported to induce neurotoxic loss of 5-HT in the hippocampus (Hadlock et al. 2011) this effect was observed following a more intense dosing schedule (four doses of 10 or 25 mg kg^{-1} given at 2 h intervals) and at a higher ambient temperature selected to ensure onset of hyperthermia, although importantly in both cases tissue was collected seven days after the final dose. Other, more recent studies have also failed to observe any neurotoxic loss of tissue monoamine levels following mephedrone administration (Baumann et al. 2012; Motbey et al. 2012; den Hollander et al. 2013).

In summary, cathinone, mephedrone and MDMA caused hyperactivity in rats with evidence of locomotor sensitisation following intermittent dosing with cathinone and mephedrone. All drugs appeared to impair NOD, although the decrease in total levels of object exploration during the familiarisation trial suggests that this may be due to a non-specific mechanism and not a deficit in visual recognition memory. Mephedrone (10 mg kg^{-1}) did selectively impair retention of fear motivated contextual memory in CER but the mechanism involved in this effect is currently unknown. There was no significant effect of the repeated intermittent dosing regimen on CER acquisition, sensorimotor gating, or brain tissue levels of dopamine or 5-HT seven days after the final injection. To conclude, it appears that while cathinone, mephedrone and MDMA may have similar mechanisms of action, the behavioural responses are clearly dependent on both the dose and frequency of administration.

Since mephedrone is often taken by previous MDMA users and can be taken concomitantly with other psychostimulants (Moore et al. 2013), the studies in the next chapter investigated the effects of previous MDMA exposure during early adolescence administration or concomitant caffeine administration on mephedrone-induced changes in LMA, NOD, temperature, elevated plus maze and PPI.

Chapter 4 Effects of MDMA pre-exposure or caffeine co-administration on the behavioural and neurochemical responses to mephedrone

4.1 Introduction

Toxicological investigations into the cause of death in mephedrone-related fatalities in the UK (2009-2011) have confirmed poly-drug intoxication in a number of cases (Winstock et al. 2011; Corkery et al. 2012). Mephedrone users often report taking other stimulants, such as alcohol, cannabis, MDMA or cocaine concurrently. For instance, in one survey the majority of mephedrone users (87% of 1506 survey participants) admitted previous or concurrent illicit use of MDMA (Carhart-Harris et al. 2011). Repeated MDMA administration to rats produces behavioural sensitisation both to MDMA itself as well as to other 'amphetamine-like' psychostimulants. This sensitisation is thought to result from enhanced dopamine release in the nucleus accumbens and striatum (Robinson and Becker 1982) and is both dose level and administration interval dependent (Kalivas et al. 1993). As previously stated, mephedrone causes some similar effects to MDMA in recreational users, including changes in thermoregulation, cognitive deficits, hallucinations, anxiety, agitation and hypertension (Dargan et al. 2010; Freeman et al. 2012) most of which have been replicated in the rodent (Meng et al. 2012; Motbey et al. 2012; den Hollander et al. 2013; Shortall et al. 2013). Although the effects of mephedrone are short term in comparison to those of MDMA and appears to have less potential long term neurotoxicity (Baumann et al. 2012; Motbey et al. 2012; den Hollander et al. 2013). Therefore the characterisation of any change in the acute effects of mephedrone after MDMA pre-exposure is worth investigation.

Although mephedrone is now banned across Europe and the USA (Dargan et al. 2011; Gershman and Fass 2012), analysis of 'legal high' products after the UK ban on mephedrone found that these products contained mephedrone along with other psychostimulants, such as caffeine (Brandt et al. 2010; Davies et al. 2010; Rosenauer et al. 2013). Caffeine is known to increase the toxicity of MDMA in rats, characterised by seizures, hyperthermia, tachycardia and lethality (for review see Vanattou-Saïfoudine et al., 2012). The increase in caffeinated energy drink consumption also means that mephedrone users may intentionally ingest high levels of caffeine with mephedrone (Reissig et al. 2009). It is

therefore worth investigating whether caffeine co-administration exacerbates the acute effects of mephedrone.

Prior to examining the influence of MDMA or caffeine on responses to mephedrone, a preliminary dose-response study was conducted to allow selection of a submaximal mephedrone dose and ensuring any synergistic interaction with the other drugs could be detected. Rats received 10 or 30 mg kg^{-1} of mephedrone and LMA was recorded for 60 min post-injection, at the end of which a single rectal temperature measurement was made. On the basis of this study a 10 mg kg^{-1} dose of mephedrone was chosen for the further two studies in this chapter, which investigated the effects of MDMA pre-exposure during early adolescence or concomitant caffeine administration on the behavioural responses of rats to mephedrone during adulthood. In the MDMA study, during the pre-exposure phase involved a single i.p. injection of saline vehicle or MDMA (5 mg kg^{-1}) once daily for seven days, followed by a seven day washout period before behavioural testing. This dosing schedule is known to produce locomotor sensitisation to MDMA (Aberg et al. 2007) and in the current study rats received a single injection of vehicle, MDMA (5 mg kg^{-1}) or mephedrone (10 mg kg^{-1}) on each day of behavioural testing. In the caffeine study, rats received i.p. saline vehicle, mephedrone (10 mg kg^{-1}) or caffeine (10 mg kg^{-1}) alone or in combination on each behavioural test day. This dose of caffeine was selected as it alters the locomotor activity and temperature responses to MDMA in the rat (Vanattou-Saifoudine et al. 2010). The dose schedule described in section 3.3.3 was used for the behavioural phase of both studies, where rats received two injections per week over three weeks, and Chapter 3 showed that this chronic intermittent administration of 10 mg kg^{-1} of mephedrone alone produced marked pharmacological and behavioural effects in the rat without any accompanying long-term monoamine neurotoxicity. In both the MDMA and caffeine studies the hypothalamus, frontal cortex, hippocampus and hypothalamus (selected because of their known sensitivity to the acute effect of MDMA on monoamine release) were collected 60 min (MDMA study) or 7 days (study caffeine study) after the final mephedrone injection to measure any changes in dopamine, 5-HT or their major metabolites.

4.2 Aims

The main aims of this set of experiments were to:

(1) investigate the effects of MDMA administration to rats on subsequent mephedrone or MDMA challenge;

(2) investigate the effects of concomitant caffeine and mephedrone administration on various behavioural responses to mephedrone;

(3) identify any neurochemical changes that occur in specific brain regions either 60 min (study two) or seven days (study three) after the final mephedrone injection.

4.3 Materials and Methods

4.3.1 Animals

Experimentally naïve young-adult male Lister hooded rats (210-250g at start of behavioural testing; Charles River UK) were housed in groups of four in wire-top cages (in Scantainer ventilated cabinets for the caffeine study) under constant environmental conditions as described in section 2.3.1. All experiments were performed during the light phase between 9.00 h and 16.00 h. All procedures were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and ARRIVE guidelines, with approval of the University of Nottingham Local Ethical Committee. The doses of drugs and behavioural schedule were chosen to comply with the three R's of humane animal testing. In all experiments, rats were allocated to a treatment group using a pseudorandom design but initial body weights were checked to ensure no basal weight differences between groups. The body weight of each rat was taken before the first injection and then monitored throughout the remainder of the experiment.

4.3.2 Drugs

Caffeine ReagentPlus was purchased from Sigma Aldrich, UK. (±)-MDMA-HCl was obtained from Tocris Bioscience, UK and (±)-mephedrone-HCl was purchased from Ascent Scientific, UK. All drugs were dissolved in saline vehicle (0.154 M) and doses are quoted as the salt.

4.3.3 Experimental design

4.3.3.1 Study one: Mephedrone dose response

LMA was performed as described in section 3.3.3.2. Rectal and tail temperatures were taken once immediately at the end of locomotor

recording (60 min post-injection), using the equipment described in section 2.3.3.1.

4.3.3.2 Study two: Effects of MDMA pre-exposure on mephedrone-induced changes to behaviour and temperature

To examine the effects of MDMA pre-exposure on the response to subsequent mephedrone administration, rats (n=8 per treatment group) received i.p. injections of saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) once daily for seven days (1-7), followed by a seven day washout period (days 8-14). Rats were subsequently challenged with saline vehicle (V, 1 ml kg^{-1}), MDMA (5 mg kg^{-1}) or mephedrone (Meph, 10 mg kg^{-1}) on two consecutive days a week for three weeks (days 15, 16, 22, 23, 29 and 30), to mimic typical patterns of weekend recreational use in humans (see Fig 4.1 for a summary of the dose schedule). On challenge injection days, rats were assessed for LMA (days 15 and 29), NOD (day 16), elevated plus maze behaviour (day 22), rectal and tail temperature changes (day 23) or PPI (day 30). Brain regions were collected 60 min after the final challenge injection for quantification of monoamine neurotransmitters and their major metabolites by HPLC-ED.

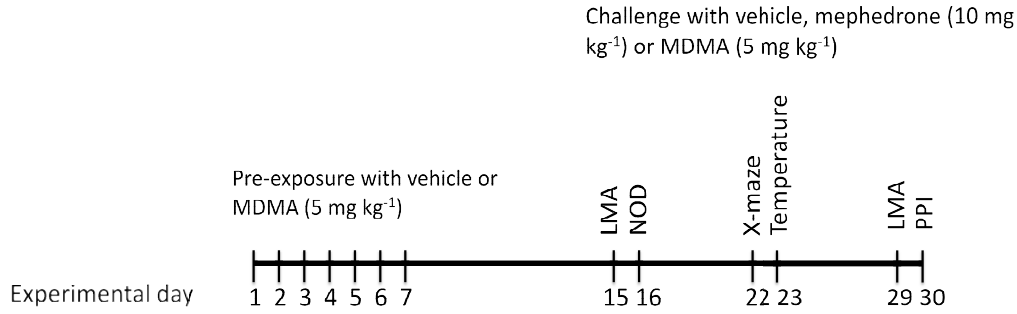
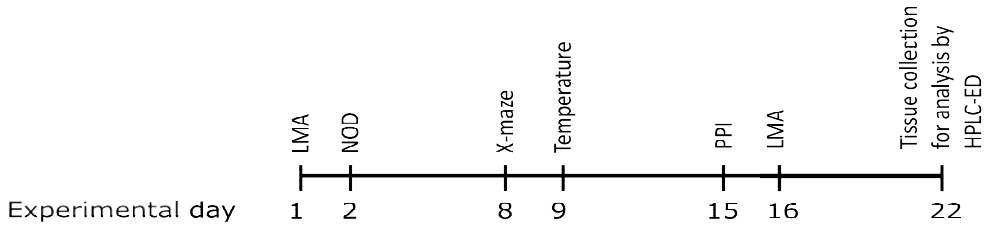


Figure 4.1 Summary of the experimental protocol for the MDMA study.

Adolescent male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) once daily for seven days (pre-exposure period) followed by a 7 day washout period. Rats subsequently received an acute challenge of either i.p. saline vehicle (V, 1 ml kg^{-1}), (±)-MDMA-HCl (5 mg kg^{-1}) or (±)-mephedrone-HCl (Meph, 10 mg kg^{-1}) on two consecutive days a week for three weeks resulting in six pre-exposure and challenge combinations abbreviated as follows V+V, V+MDMA, V+Meph, MDMA+V, MDMA+MDMA or MDMA+Meph. Locomotor activity (LMA, day 15 and 29), novel object discrimination (NOD, day 16), elevated plus maze behaviour (x-maze, day 22), rectal and tail temperature (day 23) and prepulse inhibition of the acoustic startle response (PPI, day 30) responses were measured on each injection day.

4.3.3.3 Study three: Effects of concomitant caffeine on mephedrone-induced changes to behaviour and temperature

In order to examine the effects of concomitant caffeine and mephedrone administration, rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}), mephedrone (Meph, 10 mg kg^{-1}), caffeine (Caff, 10 mg kg^{-1}) or a combination of caffeine and mephedrone (Caff+Meph) in the same injection twice weekly on consecutive days for three weeks to mimic weekend recreational use in humans (see Fig 4.2 for a summary of the dose schedule). On each injection day rats were assessed for LMA (day 1 and 16), NOD (day 2), elevated plus maze behaviour (day 8), rectal temperature changes (day 9) or PPI (day 15). Seven days after the final injection, brain regions were collected for quantification of monoamine neurotransmitters and their major metabolites by HPLC-ED.



4.2 Summary of the experimental protocol for the caffeine study

Adolescent male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}), mephedrone (10 mg kg^{-1}), caffeine (10 mg kg^{-1}), or a combination of both mephedrone and caffeine in the same injection on two consecutive days a week for three weeks. Locomotor activity (LMA, day 1 and 16), novel object discrimination (NOD, day 2), elevated plus maze (x-maze, day 8), rectal and tail temperature (day 9) and prepulse inhibition of the acoustic startle response (PPI, day 15) responses were measured on each injection day.

4.3.4 Behavioural testing

The sequence of behaviours in these studies were designed in order of least to most aversive in order to limit the effects of the previous behavioural paradigm test whilst minimising animal use to comply with the 3R's. All apparatus used in each study were cleaned with 20% ethanol between tests to remove any odour cues.

4.3.4.1 Locomotor activity

Changes in LMA were assessed on days 15 and 29 of the MDMA cross-sensitisation study and days 1 and 16 of the caffeine study as described in section 3.3.4.

4.3.4.2 Novel object discrimination

NOD was assessed on day 16 of the MDMA cross-sensitisation study and on day 2 of the caffeine study as described in section 3.3.5.

4.3.4.3 Elevated plus maze

Elevated plus maze was performed on day 22 of the MDMA cross-sensitisation study and day 8 of the caffeine study to determine any drug-induced alteration in response to mild aversive environment, using a previously described apparatus (Bull et al. 2003). The maze (black Perspex) consisted of four arms (45 cm long) arranged at right angles around a central square (10 cm x 10 cm) and elevated 70 cm above the floor. The arms consisted of two closed arms with 10 cm high walls and two open arms with no walls. Rats were injected and 30 min later placed in the centre of the maze, facing a closed arm. Light intensity was 20 lux on the closed arms and 60 lux on the open arms. Exploration was recorded for 5 min using Ethovision XT 7 software. Measures derived were number of open and closed arm entries, total time spent in open and closed arms, percentage open arm entries ([open arm entries/total arm entries] x 100), time and percentage time spent in open arms ([time spent in open arms/total time spent in all arms] x 100). Frequency of unprotected head dips over the sides of the open arms and protected stretch attends were also scored manually from video recordings using the computer keypad.

4.3.4.4 Rectal and tail temperature

Rectal and tail temperatures were recorded on day 23 of the MDMA study and day 9 of the caffeine study as described in section 2.3.3.1.

4.3.4.5 Prepulse inhibition of acoustic startle response

PPI was measured on day 30 of the MDMA cross-sensitisation study and on day 15 of the caffeine study as described in detail in section 3.3.7.

4.3.4.6 Tissue collection and neurochemical detection by HPLC-ED

Hypothalamus and right striatum, frontal cortex and hippocampus were dissected 60 min post-injection in the MDMA cross-sensitisation study and seven days after the last of six injections in the caffeine study as described in section 2.3.3.2. HPLC was also performed as described in section 2.3.3.2.

4.3.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism (v 6.02) or SPSS v 21 software. In the dose response study, LMA data was analysed by two-way repeated measures ANOVA with treatment as the between-group factor and time as the within-group factor. Rectal temperature was analysed by one-way ANOVA. In the MDMA pre-exposure study, LMA, NOD, temperature and PPI data were analysed using three-way repeated measures ANOVA with pre-exposure and challenge injections as between-group factors and time (LMA, temperature), object (NOD) or pre-pulse amplitude (PPI) as the within-group factors. X-maze data were analysed by two-way ANOVA with pre-exposure and challenge injection as factors. In the caffeine co-administration study, LMA, NOD, temperature and PPI data were analysed using three-way repeated measures ANOVA with drug injections as between-group factors and time (LMA, temperature), object (NOD) or pre-pulse amplitude (PPI) as the within-group factors. X-maze data were analysed by two-way ANOVA with drug injections as factors.

4.4 Results

4.4.1 Study one: Mephedrone dose-response

This preliminary dose-response study demonstrated a mephedrone dose-related increase in horizontal activity after habituation (data not shown) to a novel arena (Fig. 4.2A), such that there were main effects of treatment ($F_{(2,15)}$=25.96, p<0.001) and time ($F_{(11,165)}$=14.24, p<0.001) as well as a significant treatment x time interaction ($F_{(22,165)}$=3.85, p<0.001). Consistent with previous studies 10 mg kg^{-1} mephedrone elevated ambulation above control values from 10 min post-injection (p<0.001) with a return to baseline levels by 55 min. The higher (30 mg kg^{-1}) dose also elevated ambulation from 10 min post-injection, but in the case there was no return to control levels by the end of the 60 min monitoring period. In addition, activity was significantly higher than that following the lower 10 mg kg^{-1} dose at 10, 15, 35, 45 and 55 min post-injection confirming that 10 mg kg^{-1} mephedrone produces sub-maximal effects on LMA.

Mephedrone also produced a dose-related decrease in both rectal ($F_{(2,15)}$=14.32, p<0.001) and tail temperature ($F_{(2,15)}$=1.85, p<0.001; Fig. 4.2B). Both doses of mephedrone significantly decreased tail temperature 60 min post-injection (p<0.001), however only the higher 30 mg kg^{-1} dose produced a decrease in rectal temperature at the same time point (p<0.001), and in each case the temperature in the 30 mg kg^{-1} treated group was significantly lower than that of the 10 mg kg^{-1} treated group (p<0.05-0.001). Again confirming the effect of 10 mg kg^{-1} mephedrone was sub-maximal and therefore suitable to use in the MDMA pre-exposure and caffeine co-administration studies.

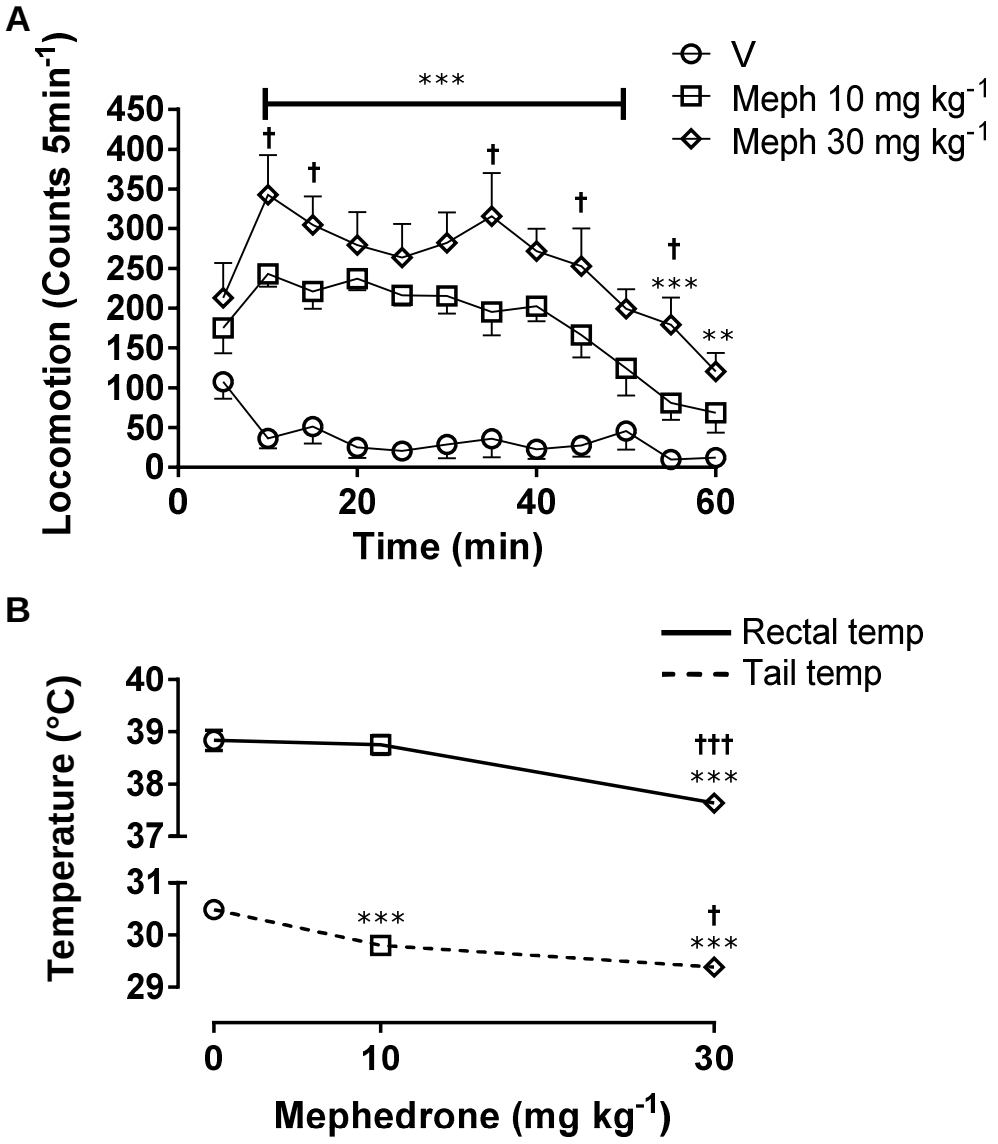


Figure 4.3 Acute mephedrone administration caused dose-related locomotor hyperactivity and hypothermia.

Mean ± SEM (**A**) locomotor activity, expressed as cumulative horizontal ambulation counts over consecutive 5 min epochs for the 60 min post-injection period, and (**B**) rectal and tail temperature (°C) measured 60 min post-injection in adult male Lister hooded rats (n=6 per group) which were habituated to the LMA boxes for 60 min and then received single i.p. injection of saline vehicle (V, 1 ml kg^{-1}) or (±)-mephedrone-HCl (Meph, 10 or 30 mg kg^{-1}) on **p<0.01, ***p<0.001 compared to saline vehicle, where the bar indicates a significant difference between vehicle and both doses of mephedrone. $^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$ 30 mg kg^{-1} mephedrone compared to 10 mg kg^{-1} mephedrone; multiple comparison post-hoc following two-way repeated measures ANOVA for LMA or following one-way ANOVA for temperature data.

4.4.2 Study two: Effects of MDMA pre-exposure on mephedrone-induced changes to behaviour and temperature

4.4.2.1 Body weight

Irrespective of treatment all rats gained weight throughout the experiment, such that there was a main effect of time ($F_{(5,210)}$=2633, p<0.001), but no effect of pre-exposure ($F_{(1,42)}$=0.42, p>0.05) or challenge treatments ($F_{(2,42)}$=0.20, p>0.05), nor any pre-exposure x time ($F_{(5,210)}$=0.58, p>0.05), challenge x time ($F_{(10,210)}$=0.27, p>0.05), pre-exposure x challenge ($F_{(2,42)}$=0.80, p>0.05), or pre-exposure x challenge x time ($F_{(10,210)}$=1.50, p>0.05, Fig 4.3) interactions.

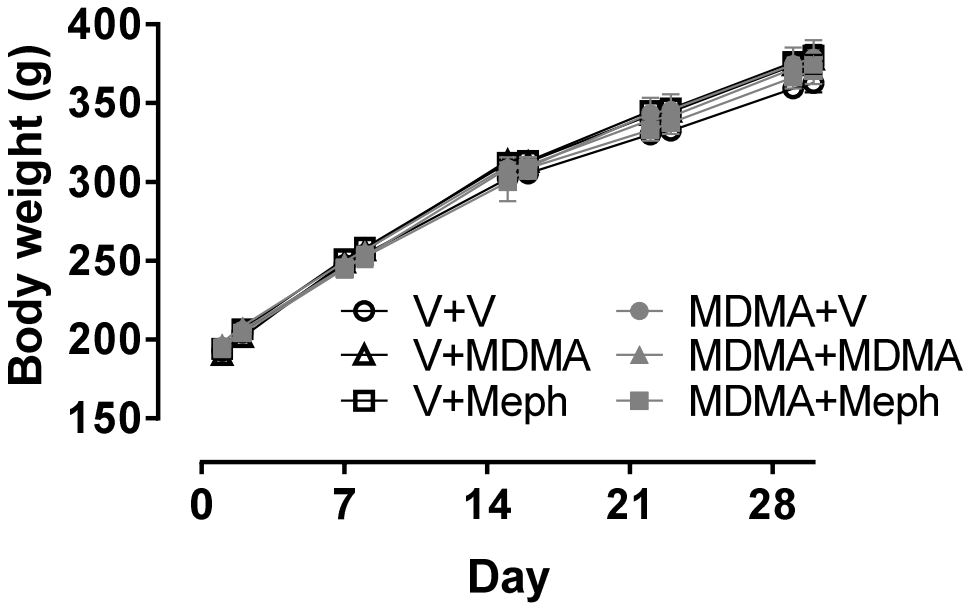


Figure 4.4 Drug treatment did not affect body weight for the duration of the MDMA pre-exposure study.

Adult male Lister hooded rats (n=8 per treatment) displayed an increase in body weight (g, mean ± SEM) throughout the experiment, which was unaffected by pre-exposure to saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) on days 1-7 of the experiment, or subsequent twice weekly administration of saline vehicle (1 ml kg^{-1}, V+V or MDMA+V), MDMA (5 mg kg^{-1}, V+MDMA or MDMA+MDMA) or mephedrone (10 mg kg^{-1}, V+Meph or MDMA+Meph) on days 15, 16, 22, 23, 28 and 29.

4.4.2.2 Locomotor activity

Habituation to the activity boxes prior to the first challenge injection on day 15 was confirmed by a decline in horizontal locomotion over the 60 min pre-injection period (data not shown but consistent with section 3.4.2) and there were no between-group differences in rats pre-exposed to vehicle or MDMA. Post-injection there were significant main effects of challenge injection ($F_{(2,42)}$=21.42, p<0.001), time ($F_{(11,462)}$=16.31, p<0.001) as well as a challenge injection x time interaction ($F_{(22,462)}$=4.75, p<0.001), Fig 4.4A, B) but no main effect of MDMA pre-exposure ($F_{(1,42)}$=2.41, p>0.05), nor any pre-exposure x time ($F_{(11,462)}$=1.09, p>0.05), pre-exposure x challenge injection ($F_{(2,42)}$=1.05, p>0.05), or pre-exposure x challenge injection x time interaction ($F_{(22,462)}$=0.72, p>0.05). Bonferroni multiple comparisons post-hoc tests showed that although MDMA challenge appeared to have increased activity in the vehicle pre-exposed group this did not reach statistical significance (Fig 4.4A). However, in MDMA pre-exposed rats MDMA challenge caused a significant increase in locomotion from 10-45 min and at 55 min compared to vehicle challenge to vehicle pre-exposed rats (p<0.05-0.001) and from 15-55 min compared to vehicle challenge to MDMA pre-exposed rats (Fig 4.4A). In vehicle pre-exposed rats mephedrone challenge caused significant hyperactivity at 10, 20 and 30 min post-injection compared to vehicle challenge to vehicle pre-exposed rats, while mephedrone challenge to MDMA pre-exposed rats caused hyperactivity from 10-30 min post-injection compared to vehicle challenge to vehicle pre-exposed rats and from 20-40 min compared to vehicle challenge in MDMA pre-exposed rats (Fig 4.4B). Despite the apparent increases in responses to both MDMA and mephedrone in MDMA pre-exposed rats, there was no significant difference in horizontal locomotion following MDMA challenge to vehicle or MDMA pre-exposed groups (Fig 4.4A) nor any difference in horizontal locomotion following mephedrone challenge to vehicle or MDMA pre-exposed groups (Fig 4.4B) at any time point.

As on day 15, habituation to the activity boxes prior to the fifth challenge injection on day 29 was confirmed by a decline in horizontal ambulatory activity over the 60 min pre-injection period (data not shown). Both MDMA (Fig 4.4C) and mephedrone (Fig 4.4D) challenge again caused significant

hyperactivity from 20 and 15 min post-injection respectively, which was sustained for the duration of the recording period for both compounds in both vehicle and MDMA pre-exposed rats (challenge $F_{(2,42)}=67.6$, $p<0.001$; time $F_{(11,462)}=7.73$, $p<0.001$, challenge injection x time interaction $F_{(22,462)}=10.28$, $p<0.001$). MDMA pre-exposure did not alter the hyperactivity observed following MDMA or mephedrone challenge, such that there no main effect of pre-exposure ($F_{(1,42)}=0.15$, $p>0.05$), nor any pre-exposure x challenge injection ($F_{(2,42)}=0.65$, $p>0.05$), or pre-exposure x challenge injection x time interactions ($F_{(22,462)}=1.06$, $p>0.05$).

To further examine the effect of MDMA pre-exposure on LMA following mephedrone or MDMA challenge, total cumulative horizontal locomotion counts in the 60 min following mephedrone or MDMA challenge were analysed. There were main effects of pre-exposure ($F_{(1,42)}=0.48$, $p>0.05$), challenge injection ($F_{(2,42)}=52.50$, $p<0.001$) and day ($F_{(1,42)}=46.38$, $p<0.001$) on total cumulative LMA counts on both LMA test days, as well as pre-exposure x day ($F_{(1,42)}=4.28$, $p<0.05$), challenge injection x day ($F_{(2,42)}=11.39$, $p<0.001$), pre-exposure x challenge injection ($F_{(2,42)}=0.12$, $p>0.05$) and pre-exposure x challenge injection x day interactions ($F_{(2,42)}=3.38$, $p<0.05$, Table 4.1). Mephedrone increased total LMA counts in both vehicle and MDMA pre-exposed rats on both test days. MDMA increased total LMA counts in MDMA pre-exposed rats, but not vehicle pre-exposed rats on day 15 and in both vehicle and MDMA pre-exposed rats on day 29. Total LMA counts for mephedrone were also significantly higher on day 29 than day 15 in both pre-exposed groups, while MDMA displayed greater horizontal locomotor activity counts on day 29 compared to day 15 in the vehicle pre-exposed rats only.

There were no significant main effects of MDMA pre-exposure or challenge injection on total rearing or fine movement counts following the final challenge injection (data not shown).

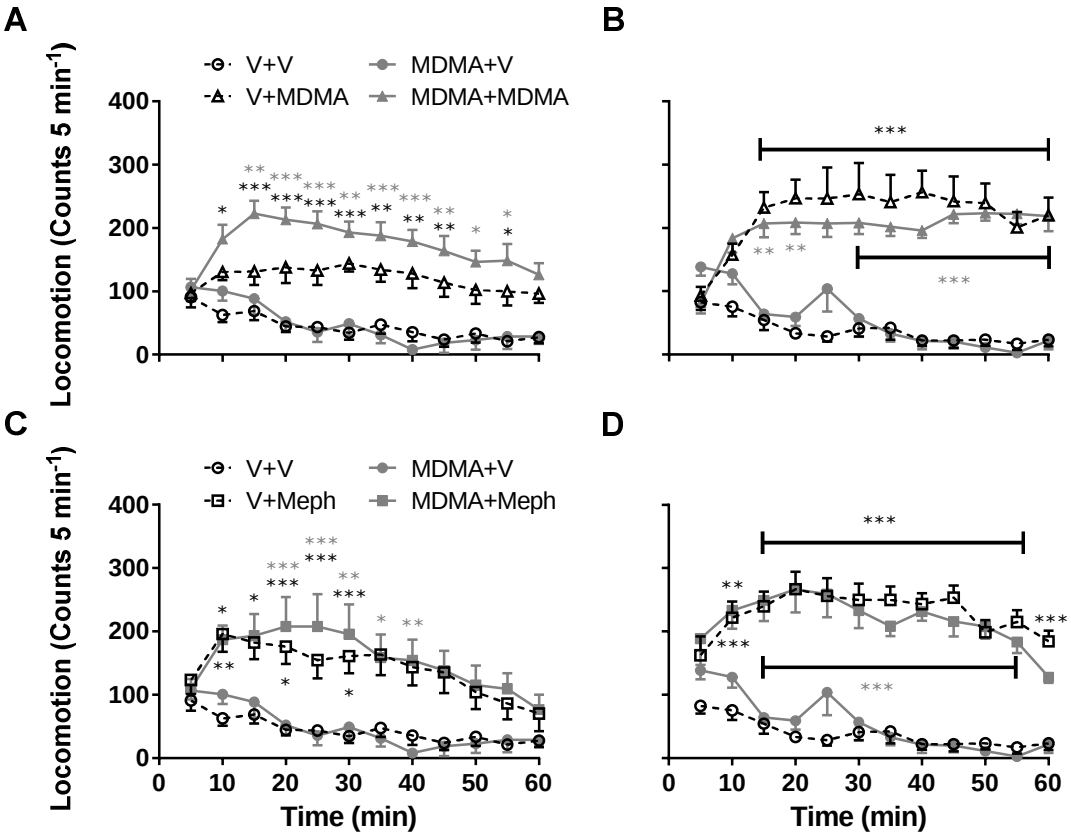


Figure 4.5 MDMA pre-exposure did not alter mephedrone-induced hyperactivity.

Adolescent male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) once daily for seven days (pre-exposure period) followed by a 7 day washout. Rats subsequently received an intermittent challenge of either i.p. saline vehicle (1 ml kg^{-1}), (**A**, **C)** (±)-MDMA HCl (5 mg kg^{-1}) or (**B**, **D**) (±)-mephedrone HCl (meph, 10 mg kg^{-1}) on two consecutive days a week for three weeks. Locomotor activity was measured on days (**A**, **B**) 15 and (**C**, **D**) 29, following the first and fifth challenge injections. The pre-exposure and challenge combinations are abbreviated as follows V+V, V+MDMA, V+Meph, MDMA+V, MDMA+MDMA or MDMA+Meph. *p<0.05, **p<0.01, ***p<0.001 compared to V+V; *p<0.05, **p<0.01, ***p<0.001 compared to MDMA+V, Bonferroni post-hoc following three-way repeated measures ANOVA. The bars on the graphs indicate significance at these time points for (**C**) V+MDMA and MDMA+MDMA and (**D**) V+Meph and MDMA+Meph. For clarity of presentation MDMA and mephedrone challenge on both days are represented in separate panels but statistical analyses were performed collectively on all groups for each day.

4.1 MDMA pre-exposure had no effect on mephedrone-induced increases in total cumulative LMA counts on day 15 or 29.

Treatment	Day 15	Day 29
V+V	533 ± 74	466 ± 73
V+Meph	1695 ± 300**	2742 ± 213***†††
V+MDMA	1447 ± 181	2629 ± 319***†††
MDMA+V	569 ± 106	660 ± 110
MDMA+Meph	1851 ± 328******	2605 ± 235******††
MDMA+MDMA	2071 ± 168******	2381 ± 153******

Adolescent male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) once daily for seven days (pre-exposure period) followed by a 7 day washout. Rats subsequently received an intermittent challenge of either i.p. saline vehicle (1 ml kg^{-1}), (±)-MDMA HCl (5 mg kg^{-1}) or (±)-mephedrone HCl (meph, 10 mg kg^{-1}) on two consecutive days a week for three weeks. Locomotor activity was measured on days 15 and 29 following the first and fifth challenge injections. The pre-exposure and challenge combinations are abbreviated as follows V+V, V+MDMA, V+Meph, MDMA+V, MDMA+MDMA or MDMA+Meph. **p<0.01, ***p<0.001 compared to V+V on the same LMA day; ***p<0.001 compared to MDMA+V on the same LMA day; ††p<0.01, †††p<0.001 compared to the same treatment on day 15, Bonferroni multiple comparisons post-hoc following three-way repeated measures ANOVA.

4.4.2.3 Novel object discrimination

NOD was assessed following the second challenge injection (day 16). There was no spatial preference for either object during the familiarisation trial for any treatment group. However, there was a significant main effect of challenge injection on time spent exploring the two identical objects during the familiarisation trial (challenge: $F_{(2,42)}=18.23$, $p<0.001$, object: $F_{(1,42)}=12.62$, $p<0.001$, data not shown), which resulted from both mephedrone and MDMA challenge reducing total object exploration, compared to vehicle challenge in vehicle or MDMA pre-exposed controls. There was no significant pre-exposure x challenge injection x object interaction on the profile of novel and familiar object exploration in the choice trial ($F_{(2,42)}=1.63$, $p>0.05$, data not shown). Similarly there was no significant effect of pre-exposure or challenge injection on the derived choice trial discrimination ratio (pre-exposure x challenge interaction: $F_{(2,42)}=2.80$, $p>0.05$, Fig 4.5).

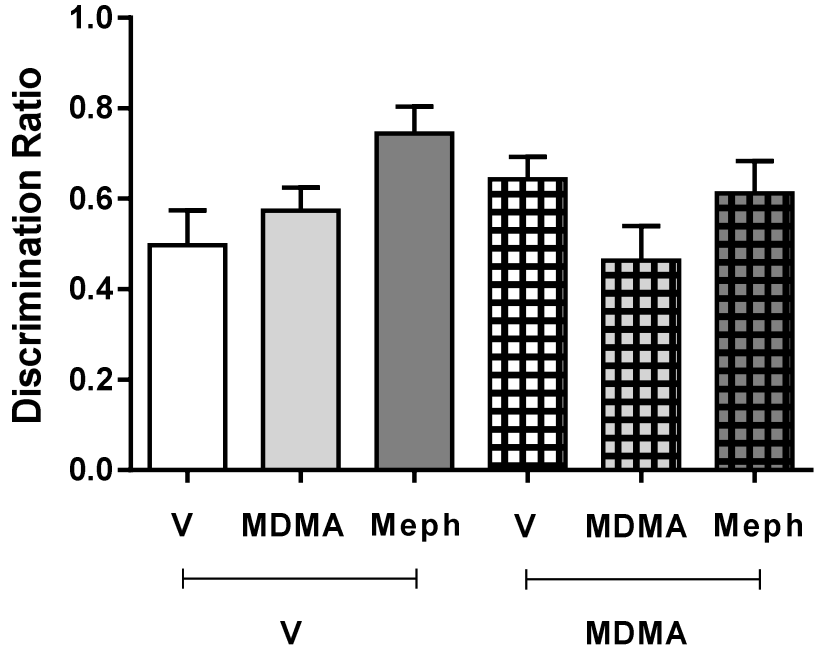


Figure 4.6 Mephedrone and MDMA had no significant effect on the NOD choice trial discrimination ratio.

Adolescent male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) once daily for seven days (pre-exposure period) followed by a 7 day washout period. Rats subsequently received an intermittent challenge of either i.p. saline vehicle (1 ml kg^{-1}), (**A**, **B**) (±)-MDMA-HCl (5 mg kg^{-1}) or (**C**, **D**) (±)-mephedrone-HCl (meph, 10 mg kg^{-1}) on two consecutive days a week for three weeks. Novel object discrimination was measured on d 16 following the second challenge injection. The pre-exposure and challenge combinations are abbreviated as follows V+V, V+MDMA, V+Meph, MDMA+V, MDMA+MDMA or MDMA+Meph.

4.4.2.4 Elevated plus maze

Following the third challenge injection (day 22), both MDMA and mephedrone increased 'anxiety-like' behaviour on the x-maze. MDMA and mephedrone challenge injections reduced the percentage time spent in the more aversive open arms of the plus maze (pre-exposure: $F_{(1,42)}=0.17$, $p>0.05$; challenge injection: $F_{(2,42)}=10.15$, $p<0.001$; pre-exposure x challenge injection interaction: $F_{(2,42)}=3.71$, $p<0.05$, Fig 4.6A) and the number of exploratory head dips over the sides of the open arms (pre-exposure: $F_{(1,42)}=1.65$, $p>0.05$; challenge injection: $F_{(2,42)}=10.02$, $p<0.001$; pre-exposure x challenge injection interaction: $F_{(2,42)}=3.59$, $p<0.05$, Fig 4.6B). However, the 'anxiety-like' effects of MDMA challenge were only observed in vehicle and not MDMA pre-exposed rats. In contrast, the 'anxiety-like' effect of mephedrone challenge on percent open arm time was only evident in MDMA pre-exposed rats, but the effect on head dips was evident irrespective of pre-exposure. Stretch attends were not observed.

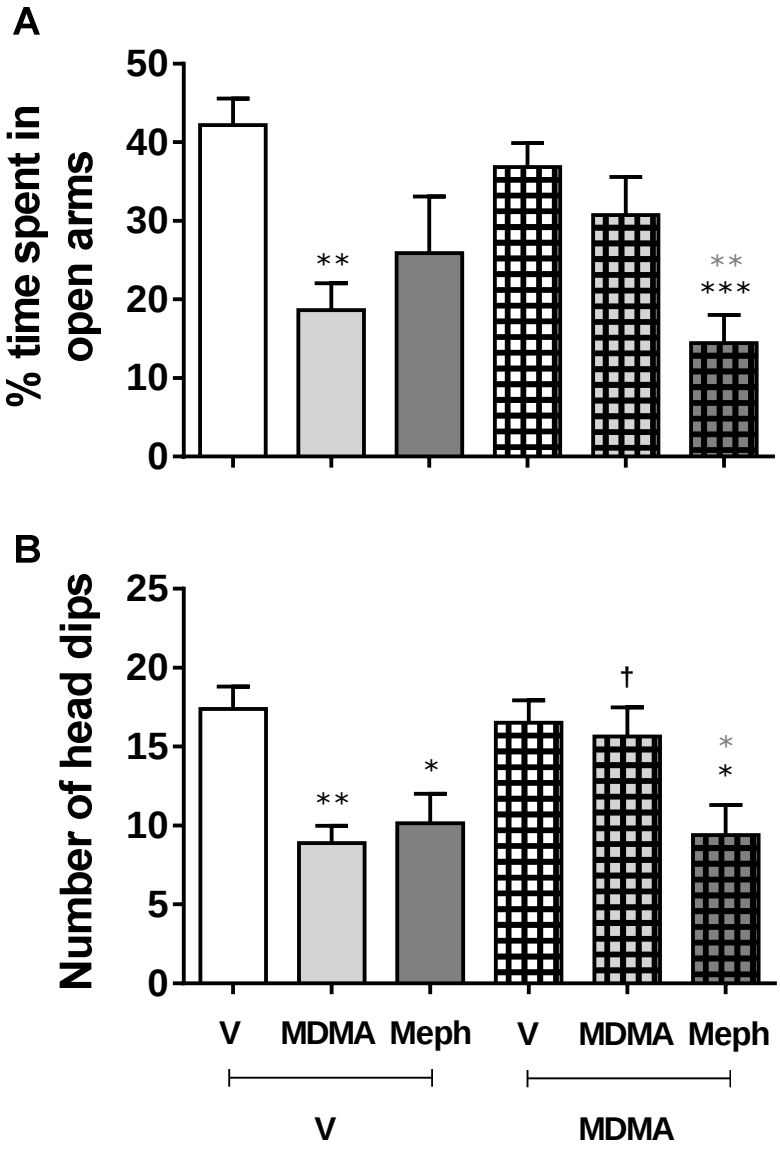


Figure 4.7 MDMA pre-exposure reversed 'anxiety-related' behaviour on the elevated plus maze following MDMA challenge.

Adolescent male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) once daily for seven days (pre-exposure period) followed by a 7 day washout period. Rats subsequently received an intermittent challenge of either i.p. saline vehicle (1 ml kg^{-1}), (±)-MDMA-HCl (5 mg kg^{-1}, A, B) or (±)-mephedrone-HCl (meph, 10 mg kg^{-1}, C, D) on two consecutive days a week for three weeks. (**A**) Percentage open arm entries and (**B**) unprotected head dips on the elevated plus maze were measured on day 22, following the third challenge injection. The pre-exposure and challenge combinations are abbreviated as follows V+V, V+MDMA, V+Meph, MDMA+V, MDMA+MDMA or MDMA+Meph. *p<0.05, **p<0.01, ***p<0.001 compared to V+V; *p<0.05, **p<0.01 compared to MDMA+V; †p<0.05 compare to V+MDMA, Bonferroni post-hoc following three-way repeated measures ANOVA.

4.4.2.5 Rectal and tail temperature

There was no significant difference in initial rectal and tail temperatures 40 min prior to the fourth challenge injection on day 23 (data not shown). There was a significant main effect of pre-exposure ($F_{(1,42)}$=6.39, $p<0.05$), challenge injection: ($F_{(2,42)}$=22.71, $p<0.001$) and time ($F_{(6,252)}$= 4.16, $p<0.001$) on rectal temperature as well as a challenge x time interaction ($F_{(12,252)}$= 5.41, $p<0.001$) but no pre-exposure x challenge injection ($F_{(2,42)}$=3.10, $p=0.056$), pre-exposure x time ($F_{(6,252)}$= 1.64, $p>0.05$) or pre-exposure x challenge injection x time ($F_{(12,252)}$=1.30, $p>0.05$) interactions. MDMA challenge to individually-housed rats at normal room temperature caused a significant decrease in rectal temperature (by 1°C at 60 min) in vehicle pre-exposed rats, which was evident from 40-80 min post-injection (Fig 4.7A). This response was attenuated in MDMA pre-exposed rats (only decreasing by 0.5°C at 60 min) such that this group of rats did not display any significant change in temperature from the vehicle control groups. In contrast to previous temperature studies (Chapter 2), mephedrone challenge did not cause a significant decrease in rectal temperature in either vehicle or MDMA pre-exposed rats (Fig 4.7B). There was no significant effect of MDMA or mephedrone challenge on tail temperature (data not shown).

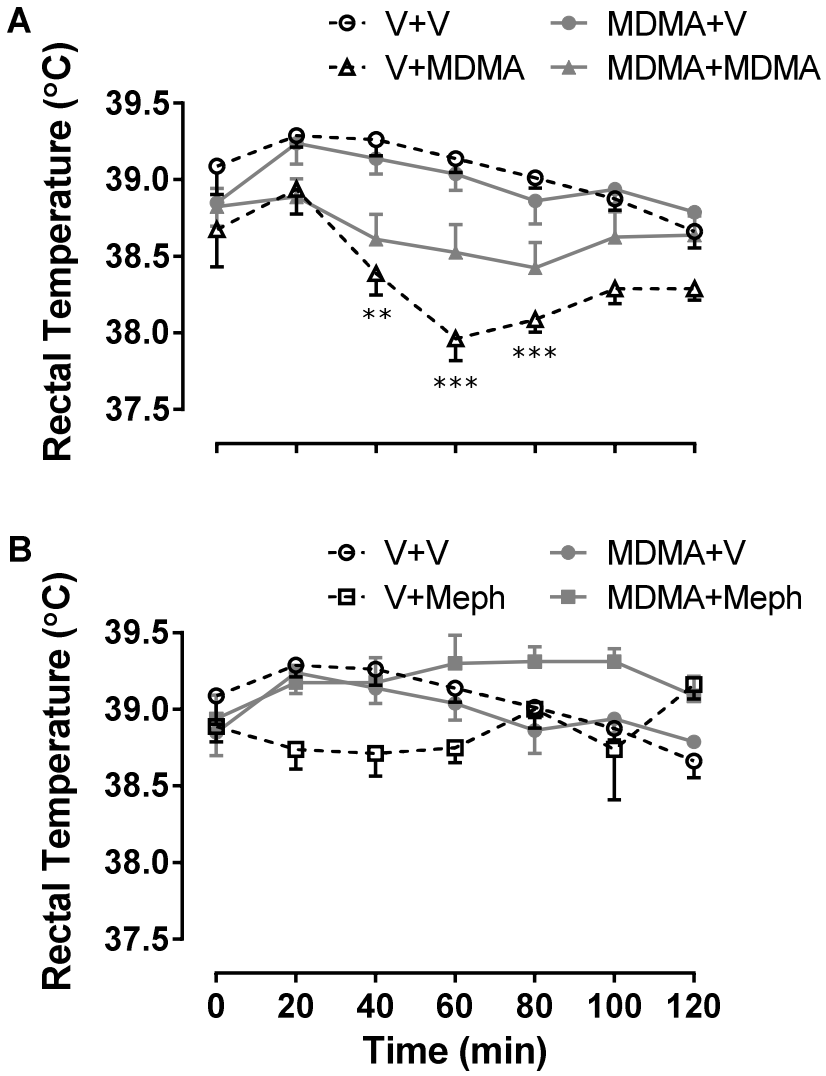


Figure 4.8 Effect of MDMA pre-exposure on subsequent MDMA and mephedrone-induced changes in temperature.

Adolescent male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) once daily for seven days (pre-exposure period) followed by a 7 day washout period. Rats subsequently received an intermittent challenge of either i.p. saline vehicle (1 ml kg^{-1}), (**A**) (±)-MDMA-HCl (5 mg kg^{-1}) or (**B**) (±)-mephedrone-HCl (meph, 10 mg kg^{-1}) on two consecutive days a week for three weeks. Rectal temperature was measured on day 23 following the fourth challenge injection. The pre-exposure and challenge combinations are abbreviated as follows V+V, V+MDMA, V+Meph, MDMA+V, MDMA+MDMA or MDMA+Meph. *p<0.05, **p<0.01, ***p<0.001 compared to V+V, Bonferroni post-hoc following three-way repeated measures ANOVA. For clarity of presentation MDMA and mephedrone challenge are represented on separate figures but statistical analyses were performed collectively on all groups on each day.

4.4.2.6 Prepulse inhibition of acoustic startle response

When PPI was assessed on day 30, following the sixth challenge injection, all groups exhibited the normal attenuation of startle response by exposure to increasing pre-pulse amplitude ($F_{(2,84)}$=74.69, p<0.001, Fig 4.8), and there was no difference in basal startle reactivity or habituation to the startle across the session in any group. There was no significant main effect of pre-exposure ($F_{(1,42)}$=0.001, p>0.05) or challenge injection ($F_{(2,42)}$=2.03, p>0.05), nor was there any pre-exposure x challenge injection x pre-pulse amplitude interaction ($F_{(2,84)}$=1.43, p>0.05) on % PPI.

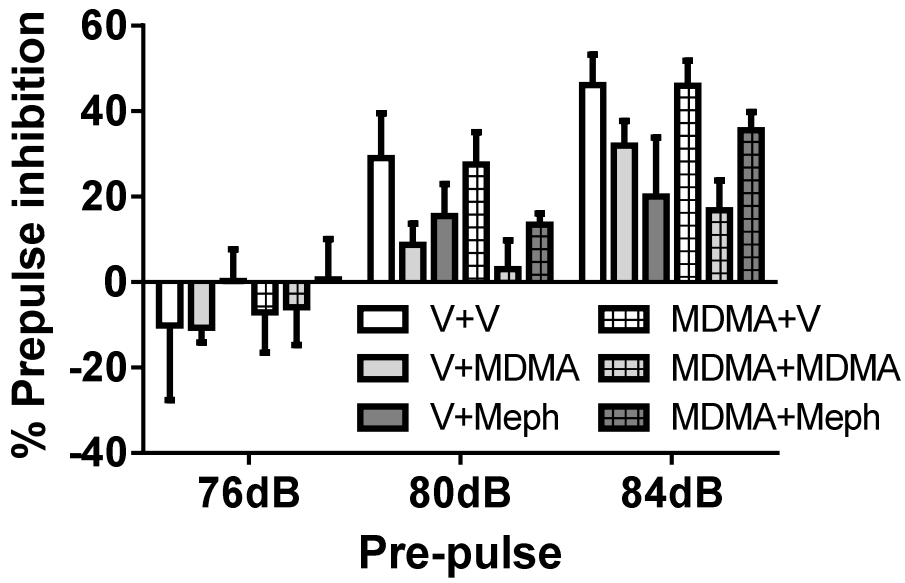


Figure 4.9 MDMA and mephedrone did not alter prepulse inhibition of the acoustic startle response.

Adolescent male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) once daily for seven days (pre-exposure period) followed by a 7 day washout period. Rats subsequently received an intermittent challenge of either i.p. saline vehicle (1 ml kg^{-1}), (**A**, **B**) (±)-MDMA-HCl (5 mg kg^{-1}) or (**C**, **D**) (±)-mephedrone-HCl (meph, 10 mg kg^{-1}) on two consecutive days a week for three weeks. Prepulse inhibition of the acoustic startle response was measured on day 30 following the sixth challenge injection. The pre-exposure and challenge combinations are abbreviated as follows V+V, V+MDMA, V+Meph, MDMA+V, MDMA+MDMA or MDMA+Meph.

4.4.2.7 Neurochemistry

There were no significant effects of repeated mephedrone or MDMA injections on the levels of dopamine or its metabolites in the frontal cortex, striatum, hippocampus or hypothalamus 60 min after the final challenge injection (data not shown). In vehicle pre-exposed rats, 5-HIAA content was reduced in the hippocampus (challenge $F_{(2,42)}=6.89$, $p<0.01$, Table 4.2) and striatum (challenge $F_{(2,40)}=3.63$, $p<0.05$) by mephedrone and MDMA respectively. In MDMA pre-exposed rats, MDMA produced a decrease in hippocampal 5-HT (challenge $F_{(2,38)}=4.42$, $p<0.05$) and 5-HIAA levels, whereas mephedrone had no significant effect on hippocampal 5-HT or 5-HIAA content in MDMA pre-treated rats. There was no main effect of pre-exposure or a pre-exposure x challenge interaction on hippocampal or striatal 5-HIAA or hippocampal 5-HT levels.

Table 4.2 MDMA and mephedrone challenge injections altered ex vivo 5-HT and 5-HIAA levels in the hippocampus and striatum.

Treatment	5-HT (pmol mg^{-1} tissue)	5-HIAA (pmol mg^{-1} tissue)
	Hippocampus	
V+V	2.33 ± 0.4	2.62 ± 0.2
V+MDMA	1.64 ± 0.3	2.09 ± 0.1
V+Meph	1.35 ± 0.3	1.80 ± 0.1 *
MDMA+V	2.23 ± 0.2	2.29 ± 0.2
MDMA+MDMA	1.24 ± 0.2 *	1.60 ± 0.2 **
MDMA+Meph	1.85 ± 0.4	2.04 ± 0.2
	Striatum	
V+V	3.75 ± 0.7	4.02 ± 0.5
V+MDMA	3.23 ± 0.6	2.92 ± 0.2 *
V+Meph	3.23 ± 0.7	3.06 ± 0.5
MDMA+V	3.58 ± 0.6	3.45 ± 0.2
MDMA+MDMA	3.94 ± 0.4	3.00 ± 0.3
MDMA+Meph	3.40 ± 0.2	2.90 ± 0.2

Post-mortem tissue 5-HT and 5-HIAA levels were measured 60 min after the last of six challenge injections (day 30) of saline vehicle (V, 1 ml kg^{-1}), MDMA (5 mg kg^{-1}) or mephedrone (Meph, 10 mg kg^{-1}) to adult male Lister hooded rats (n=8 per group) which had previously received vehicle (1 ml kg^{-1}) or MDMA (5 mg kg^{-1}) on days 1-7 followed by a washout period from days 8-14. *$p<0.05$, **$p<0.01$ compared to V+V (for vehicle pre-exposed rats) or MDMA+V (for MDMA pre-exposed rats), Bonferroni multiple comparison post-hoc following two-way ANOVA. The pre-exposure and challenge combinations are abbreviated as follows V+V, V+MDMA, V+Meph, MDMA+V, MDMA+MDMA or MDMA+Meph.

4.4.3 Study three: Effects of concomitant caffeine on mephedrone-induced changes to behaviour and temperature

4.4.3.1 Body weight

Irrespective of treatment all rats gained weight throughout the experiment such that there was a main effect of time ($F_{(5,210)}$=2632.60, p<0.001) but no effect of either drug alone (caffeine: $F_{(1,42)}$=0.04, p>0.05; mephedrone: $F_{(2,42)}$=0.20, p>0.05) nor any caffeine x mephedrone ($F_{(2,42)}$=0.80, p>0.05) caffeine x time ($F_{(5,210)}$=0.5.8, p>0.05), mephedrone x time ($F_{(10,210)}$=0.27, p>0.05) or caffeine x mephedrone x time interactions ($F_{(10,210)}$=1.50, p>0.05, Fig 4.9).

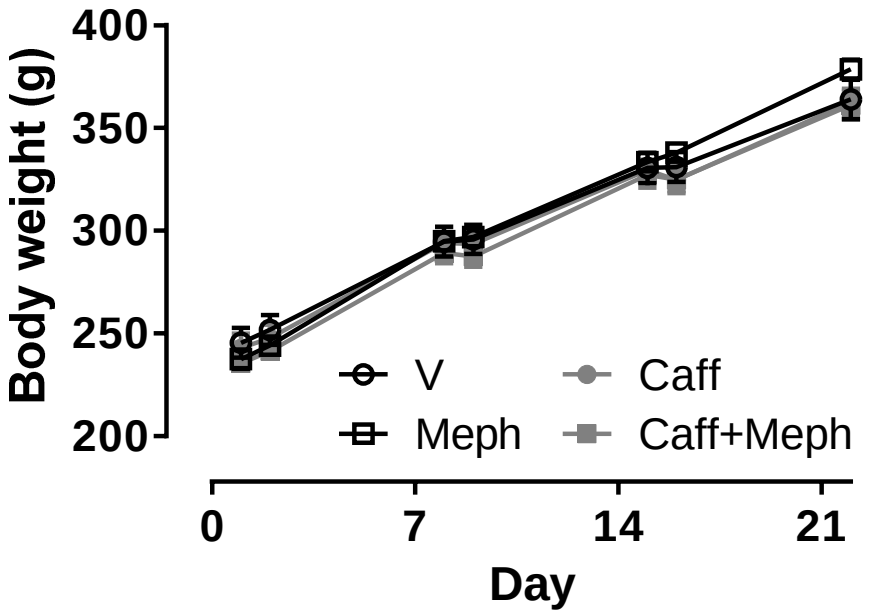


Figure 4.10 Caffeine or mephedrone administration, alone or in combination, did not affect body weight for the duration of the experiment.

Adult male Lister hooded rats (n=8 per treatment group) displayed a normal increase in body weight (g, mean ± SEM) throughout the experiment, which was unaffected by received twice weekly i.p. injections of saline vehicle (V, 1 ml kg^{-1}), mephedrone (Meph, 10 mg kg^{-1}), caffeine (Caff, 10 mg kg^{-1}) or a combination of mephedrone and caffeine on two consecutive days each week over three weeks (days 1, 2, 8, 9, 15, 16).

4.4.3.2 Locomotor activity

Habituation to the activity boxes prior to injection on both LMA days was confirmed by a decline in horizontal locomotion over the 60 min habituation period (data not shown). On day one, mephedrone, caffeine and co-administration of mephedrone and caffeine had significant main effects on ambulatory locomotion (caffeine $F_{(1,28)}=10.21$, $p<0.01$; mephedrone $F_{(1,28)}=28.91$, $p<0.001$; time $F_{(11,308)}=7.42$, $p<0.001$; caffeine x mephedrone x time interaction $F_{(11,308)}=4.16$, $p<0.001$; Fig 4.10A). Post-hoc tests showed that mephedrone alone increased horizontal locomotion above the vehicle levels from 10-40 min post-injection whereas the combination of caffeine and mephedrone increased ambulatory activity from 10-60 min post-injection with the effect being significantly greater at 60 min post-injection than in rats that received mephedrone alone.

Similar responses were observed on day 16 where mephedrone and caffeine had a significant main effect on horizontal locomotion (caffeine $F_{(1,28)}=21.53$, $p<0.001$; mephedrone $F_{(1,28)}=44.45$, $p<0.001$; time $F_{(11,308)}=7.71$, $p<0.001$; mephedrone x time interaction: $F_{(11,308)}=4.39$, $p<0.001$, Fig 4.10B). Post-hoc tests showed that, as on day 1, mephedrone alone increased horizontal locomotion above vehicle levels from 10-40 min post-injection. Also similar to day 1, concomitant caffeine and mephedrone caused a marked increase in locomotion from 5 min post-injection which was maintained for the duration of testing.

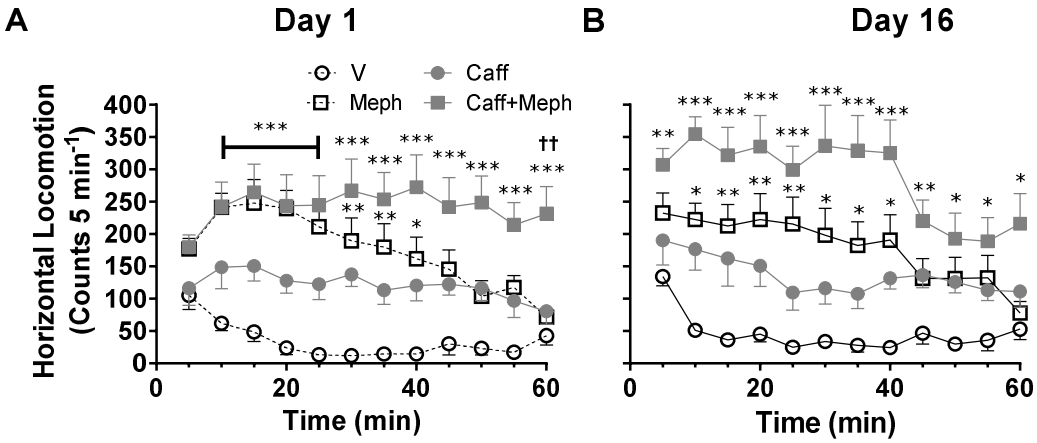


Figure 4.11 Caffeine prolonged the effect of mephedrone on locomotor activity on day 1 but not day 16 of the study.

Adult male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}), mephedrone (Meph, 10 mg kg^{-1}), caffeine (Caff, 10 mg kg^{-1}) or a combination of caffeine and mephedrone (Caff+Meph) twice weekly on two consecutive days a week for three weeks. Locomotor activity was measured on day (**A**) 1 and day (**B**) 16 following the first and sixth challenge injections. *p<0.05, **p<0.01, ***p<0.001 compared to V; ††p<0.01 compared to Meph, Bonferroni post-hoc following three-way repeated measures ANOVA. The bars indicate significance from V+V for both Meph alone and in combination with Caff.

Analysis of the total cumulative LMA counts in the 60 min post-injection period showed main effects of mephedrone such that mephedrone ($F_{(1,27)}$=33.19, p<0.001), alone and in combination with caffeine ($F_{(1,27)}$=13.79, p<0.001), increased total locomotor counts on each day compared to vehicle but there was no significant difference in horizontal locomotion counts between the first and sixth injections for any treatment group (caffeine x mephedrone x day interaction: $F_{(1,27)}$=0.11, p>0.05), Table 4.3).

4.3 Caffeine had no effect on mephedrone-induced changes in total cumulative horizontal locomotor counts on day 1 or 16.

Treatment	First injection	Sixth injection
Vehicle	407 ± 64	542 ± 38
Mephedrone	2087 ± 277***	2418 ± 374***
Caffeine	1452 ± 204	1631 ± 216
Caffeine+Mephedrone	2708 ± 487****	3241 ± 223*****

Adult male Lister hooded rats (n=8 per treatment group) received intermittent i.p. saline vehicle (1 ml kg^{-1}), mephedrone (10 mg kg^{-1}), caffeine (10 mg kg^{-1}) or a combination of caffeine and mephedrone in a single injection on two consecutive days a week for three weeks. Locomotor activity was measured on days 1 and 16 following the first and sixth injections. ***p<0.001 compared to vehicle on the same LMA day; *p<0.05, **p<0.001 compared to caffeine on the same LMA day Bonferroni multiple comparisons post-hoc following three-way repeated measures ANOVA.

There was also no significant caffeine x mephedrone x time interaction on the number of rears on each test day (day 1: $F_{(11,297)}$=1.94, p>0.05; day 16: ($F_{(11,297)}$=0.56, p>0.05) data not shown).

4.4.3.3 Novel object discrimination

NOD was assessed following the second injection (day 2). All rats explored both objects equally during the familiarisation trial (caffeine x mephedrone x object $F_{(1,28)}=0.04$, $p>0.05$), however, mephedrone treated rats spent significantly less time exploring than vehicle treated rats (mephedrone: $F_{(1,28)}=9.05$, $p<0.01$, data not shown). Vehicle treated rats successfully discriminated the novel from the familiar object during the choice trial such that there was a main effect of object ($F_{(1,28)}=4.81$, $p<0.05$), whereas treatment with caffeine or mephedrone, alone or in combination, impaired discrimination such that there was no exploratory preference for the novel object (caffeine x mephedrone x object: $F_{(1,28)}=0.47$, $p>0.05$, data not shown). However, there were no significant main effects of mephedrone ($F_{(1,28)}=0.01$, $p>0.05$), caffeine ($F_{(1,28)}=0.70$, $p>0.05$) or their combination on the discrimination ratio (caffeine x mephedrone: $F_{(1,28)}=0.47$, $p>0.05$, Fig 4.11).

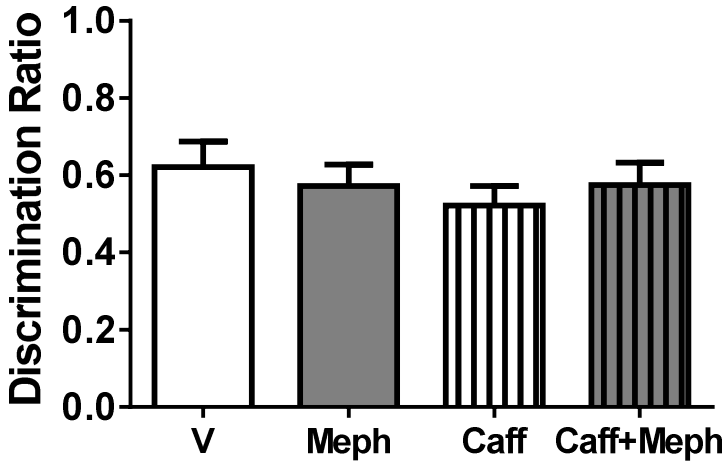


Figure 4.12 Mephedrone and caffeine had no significant effect on the NOD choice trial discrimination ratio.

Adult male Lister hooded rats (n=8 per treatment group) received intermittent i.p. saline vehicle (V, 1 ml kg^{-1}), mephedrone (Meph, 10 mg kg^{-1}), caffeine (Caff, 10 mg kg^{-1}) or a combination of caffeine and mephedrone (Caff+Meph) twice weekly on two consecutive days a week for three weeks. Novel object discrimination was measured on day 2 of the study, following the second injection.

4.4.3.4 Elevated plus maze

Although administration of caffeine or mephedrone did not significantly alter percentage entries into the open arms following the third injection (day 8; caffeine x mephedrone interaction: ($F_{(1,28)}$=0.44, p>0.05), there was a significant main effect of caffeine ($F_{(1,28)}$=4.55, p<0.05) as well as a caffeine x mephedrone interaction ($F_{(1,28)}$=4.62, p<0.05, Fig 4.12A) on percentage time spent on the open arms of the x-maze, caused by an increase in percent time on the open arms in the combined mephedrone and caffeine treated group, rather than an effect of caffeine alone.

Additionally, administration of mephedrone alone caused a decrease in the number of exploratory head dips over the sides of the open arms ($F_{(1,28)}$=7.96, p<0.01, Fig 4.12B) while simultaneously increasing the number of stretch attends ($F_{(1,28)}$=8.49, p<0.01, Fig 4.12C) compared to vehicle. Caffeine alone did not alter the number of stretch attends ($F_{(1,28)}$=1.02, p>0.05) or head dips ($F_{(1,28)}$=1.15, p>0.05) over the open arms of the maze, however concomitant caffeine and mephedrone reversed the mephedrone-induced decrease in head dip counts as well as increasing the number of stretch attends compared to vehicle (p<0.05).

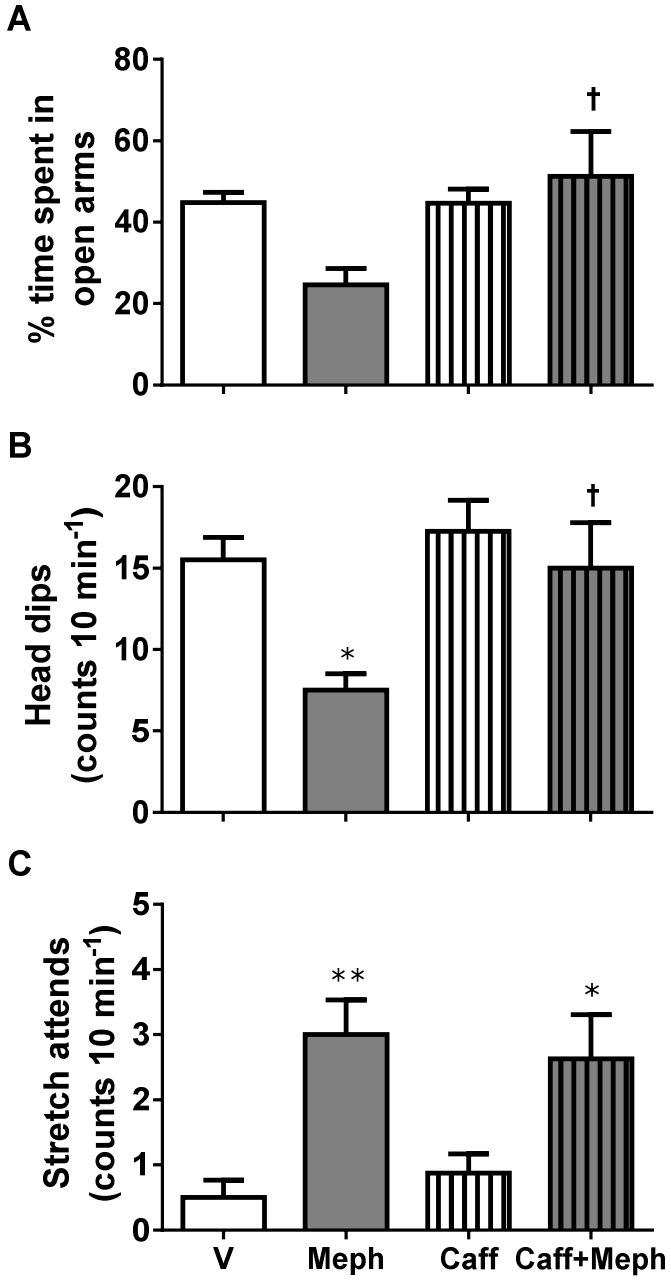


Figure 4.13 Caffeine partially reversed mephedrone-induced increases in anxiety-related behaviour on the elevated plus maze.

Adult male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (1 ml kg^{-1}), mephedrone (Meph, 10 mg kg^{-1}), caffeine (Caff, 10 mg kg^{-1}) or a combination of caffeine and mephedrone (Caff+Meph) twice weekly on two consecutive days a week for three weeks. (**A**) Percentage open arm entries, (**B**) number of unprotected head dips and (**C**) stretch attends on the elevated plus maze were measure on d 8 following the third injection. *$p<0.05$, **$p<0.01$, V; $^{\dagger}p<0.05$ compared to Meph, Bonferroni post-hoc following two-way ANOVA.

4.4.3.5 Rectal and tail temperature

There was no significant difference in initial rectal and tail temperatures 40 min prior to the fourth injection on day 9. Post-injection there was a significant main effect of caffeine and mephedrone administration on rectal temperature over time (caffeine x mephedrone x time interaction: $F_{(6,168)}=2.38$, $p<0.05$, Fig 4.13A). Post-hoc tests showed that mephedrone decreased rectal temperature from 40-60 min post mephedrone injection compared to vehicle. Caffeine alone did not significantly alter temperature, however, concomitant caffeine and mephedrone converted hypothermia to hyperthermia, where rectal temperature was significantly higher than mephedrone alone at 60-100 min and significantly higher than vehicle at 120 min post-injection. There was no significant effect of caffeine or mephedrone on tail temperature (caffeine x mephedrone x time interaction: $F_{(6,168)}=0.86$, $p>0.05$, data not shown).

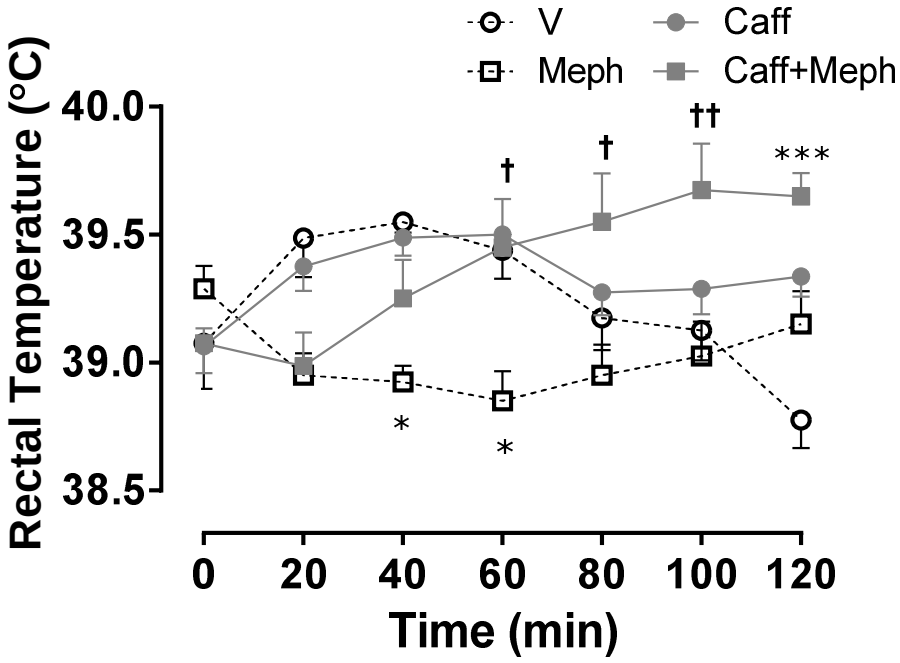


Figure 4.14 Caffeine co-administration converted mephedrone-induced hypothermia to hyperthermia.

Adult male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}), mephedrone (Meph, 10 mg kg^{-1}), caffeine (Caff, 10 mg kg^{-1}) or a combination of caffeine and mephedrone (Caff+Meph) twice weekly on two consecutive days a week for three weeks. Rectal temperature was measured on day 9 following the fourth challenge injection. *$p<0.05$, ***$p<0.001$ compared to V; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ compared to Meph, Bonferroni post-hoc following three-way repeated measures ANOVA.

4.4.3.6 Prepulse inhibition of acoustic startle response

On day 15, following the fifth injection, all treatment groups exhibited the normal attenuation of startle by exposure to increasing pre-pulse amplitude (pre-pulse: $F_{(2,48)}=60.86$, $p<0.001$). There was no difference in basal reactivity or habituation to the startle pulse alone. There was no significant effect of caffeine, mephedrone alone or in combination on percent PPI (caffeine x mephedrone x pre-pulse interaction: $F_{(2,48)}=0.09$, $p>0.05$, Fig 4.14).

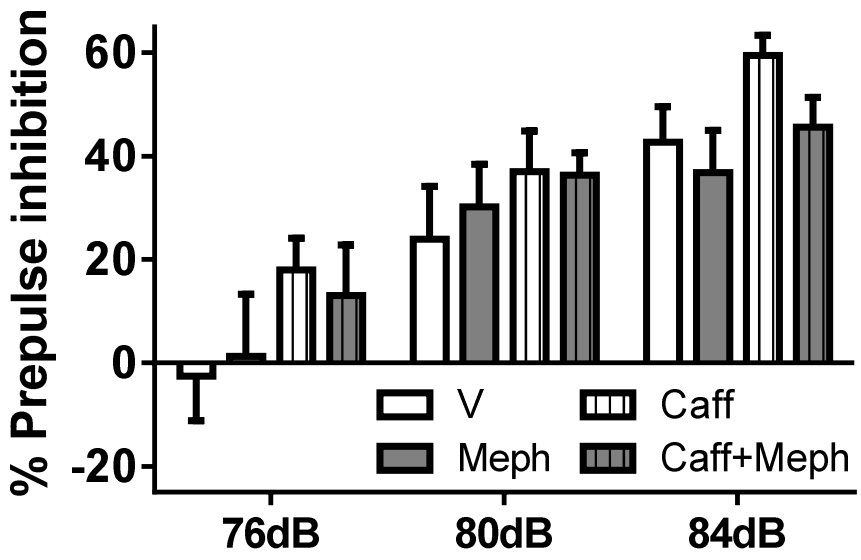


Figure 4.15 Mephedrone administration, alone or in combination with caffeine, did not alter prepulse inhibition of the acoustic startle.

Adult male Lister hooded rats (n=8 per treatment group) received i.p. saline vehicle (V, 1 ml kg^{-1}), mephedrone (Meph, 10 mg kg^{-1}), caffeine (Caff, 10 mg kg^{-1}) or a combination of caffeine and mephedrone (Caff+Meph) twice weekly on two consecutive days a week for three weeks. Prepulse inhibition of the acoustic startle response was measured on day 15 following the fifth challenge injection.

4.4.3.7 Neurochemistry

There was no significant effect of mephedrone alone, or in combination with caffeine, on *ex vivo* levels of tissue dopamine, 5-HT or their metabolites in the hypothalamus, striatum, frontal cortex or hippocampus collected seven days after the last of six injections (data not shown). Caffeine alone increased dopamine content (58.81 ± 2.5 pmol mg^{-1}, mean ± SEM) in the striatum compared to vehicle controls (34.5 ± 5.9 pmol mg^{-1}; $F_{(1,28)}$=5.38, $p<0.05$).

4.5 Discussion

The two main studies in this chapter examined firstly whether pre-exposure of adolescent rats to MDMA using a dosage schedule reported to produce locomotor sensitisation to a subsequent injection of MDMA (Aberg et al. 2007) would also enhance the behavioural responses to mephedrone and, secondly whether concurrent caffeine administration would alter the behavioural response to mephedrone. An initial dose-response study demonstrated that 10 mg kg^{-1} mephedrone produces submaximal changes in both locomotor activity and rectal and tail temperature, therefore allowing any synergistic interaction of MDMA pre-exposure or caffeine co-administration to be detected in the chosen paradigms. Pre-exposure to repeated MDMA injection enhanced the locomotor, thermoregulatory and 'anxiety-related' behavioural effects of MDMA, consistent with the development of sensitisation. Although there was no alteration in the locomotor stimulant effects of mephedrone, mephedrone-induced hypothermia was converted to mild hyperthermia by MDMA pre-exposure which may be due to cross-sensitisation. Both MDMA and mephedrone produced an anxiogenic response in the x-maze but MDMA pre-exposure only prevented the anxiogenic effect of MDMA without altering the anxiogenic effect of mephedrone. Additionally, caffeine co-administration had an additive effect on mephedrone-induced hyperactivity, converted mephedrone-induced hypothermia to hyperthermia and attenuated 'anxiety-like' behaviours on the x-maze. Finally, MDMA challenge only decreased hippocampal 5-HT in rats pre-exposed to MDMA during adolescence but MDMA pre-exposure prevented the mephedrone-induced decrease in hippocampal 5-HIAA.

Consistent with previous observations in this thesis (Chapter 3) and in other published work (Lisek et al. 2012), acute mephedrone caused significant ambulatory hyperactivity in drug naïve rats in both studies. Administration of MDMA (5 mg kg^{-1}) once daily for seven days induced locomotor sensitisation to MDMA on day 15, as indicated by the enhanced locomotor response to the first MDMA challenge in MDMA pre-exposed rats, thereby confirming the observations of Aberg et al. (2007). However, the locomotor response to either the first or fifth of six mephedrone challenge

injections was not enhanced by MDMA pre-exposure, indicating a lack of cross-sensitisation to the stimulant effects of mephedrone after MDMA. In contrast, caffeine prolonged the hyper-locomotor response to mephedrone so that activity remained elevated until the end of the 60 min test session (instead of returning to vehicle control levels after 40 min in the absence of caffeine).

MDMA impairs working memory in both humans (Bolla et al. 1998; Parrott 1998) and rats (Piper and Meyer 2004) and mephedrone has also been reported to impair working memory in human users (Freeman et al. 2012). In the MDMA pre-exposure study, mephedrone and MDMA challenge both prevented discrimination between the novel and familiar objects during the NOD choice trial, but because they also decreased total object-directed exploration during the familiarisation trial the observed reduction in object discrimination may be due to reduced attention during learning rather than a selective memory impairment, and similar findings were obtained in the mephedrone treated rats in the caffeine co-administration study.

MDMA and mephedrone can cause anxiety in human users (Parrott et al. 2000; Dargan et al. 2011) and it has also been shown that MDMA affects 'anxiety-related' behaviour in rats (Bull et al. 2003). Administration of low to medium doses of MDMA (<10 mg kg^{-1}) causes anxiogenic-like behaviour in the elevated plus maze (increased avoidance of the open arms and reduced exploratory head dips), but this effect is less marked with higher doses (Ho et al. 2004). Previous studies have shown that repeated mephedrone administration has no long-term effect on rodent 'anxiety-related' behaviour on the elevated plus maze (Motbey et al. 2012; den Hollander et al. 2013). In the MDMA pre-exposure study presented in this chapter, MDMA challenge indeed had an anxiogenic effect in vehicle pre-exposed rats, reducing the percentage time in the more aversive open arms of the maze as well as the number of exploratory head dips over the open arms. However, these effects were not seen following MDMA pre-exposure and it is possible that the combination of seven pre-exposure and three challenge MDMA injections caused sensitisation to the effects of this compound on the plus maze since increasing the dose of MDMA can attenuate the anxiogenic effect (Ho et al. 2004). In contrast, mephedrone

reduced the number of exploratory head dips in both vehicle and MDMA pre-exposed rats, while reducing the percentage time spent in the open arms of the maze in MDMA pre-exposed rats only. While MDMA pre-exposure may be attenuating mephedrone-induced anxiety-like behaviour, this attenuation was observed in one behaviour, but not the other, so it is important to not overstate this.

Caffeine administration (10 and 30 mg kg^{-1}) to rats reduces 'anxiety-related' behaviour in the elevated plus maze (Garcia et al. 2011), whilst very high doses of caffeine (100 mg kg^{-1}) increase anxiogenic-like behaviour in the maze (Braun et al. 2011). In the caffeine co-administration study, mephedrone also reduced percentage time spent in the open arms of the plus maze as well as the number of head dips, while increasing the number of stretch attends observed. Interestingly, caffeine co-administration attenuated the first two responses whilst the number of stretch attends remained unaffected. Protected stretch attends into the open arms are a risk assessment behaviour and are related to open arm avoidance, so while caffeine reduced some anxiety-related behaviour on the plus maze, it did not completely reverse the anxiogenic effects of mephedrone.

MDMA can cause hyperthermia in recreational users (Parrott 2012) which can lead to potentially fatal symptoms of heatstroke, including rhabdomylosis, myoglobinuria, liver and kidney failure (Green et al. 2004). Although the hyperthermic and long-term neurotoxic effects of MDMA are enhanced in hot room temperature conditions, the studies in this were not performed to determine such toxic effects. Additionally, co-administration of caffeine and MDMA causes a dose dependent increase in temperature in the rat, and reverses the MDMA-induced hypothermia to hyperthermia (McNamara et al. 2006; Vanattou-Saifoudine et al. 2010). It was seen in chapter 2 that acute mephedrone (10 mg kg^{-1}) and MDMA (10 mg kg^{-1}) injection both decrease rectal and tail temperature in singly housed rats at normal ambient temperatures. In the current chapter, MDMA challenge caused significant hypothermia in vehicle pre-exposed rats only. Repeated MDMA administration has a cumulative effect on temperature such that initial hypothermia is converted to hyperthermia by subsequent doses

(Rodsiri et al. 2011) and increasing the MDMA dose also transforms the hypothermic to a hyperthermic response (Docherty and Green 2010). The current attenuation of MDMA-induced hypothermia by MDMA pre-exposure is therefore more likely to reflect an enhanced response rather than developed tolerance to MDMA. While mephedrone challenge to vehicle pre-exposed rats appeared to reduce rectal temperature, this was not significant from vehicle. Interestingly, this response was not observed in MDMA pre-exposed rats. Instead this group displayed a modest hyperthermia towards the end of the recording period. This alteration probably reflects an enhanced response to mephedrone similar to that already discussed for MDMA, which may suggest some cross-sensitisation between the thermoregulatory effects of MDMA and mephedrone, although since these effects were not significant, further investigation is required. In the caffeine co-administration study reported in this chapter, mephedrone also reduced rectal temperature which was converted to hyperthermia in the presence of caffeine. This may account for the hot flushes and sweating reported by human users who are likely to be ingesting caffeine concomitantly with mephedrone (Reissig et al. 2009; Davies et al. 2010; Rosenauer et al. 2013). As previously mentioned, this is similar to what has been observed following MDMA and caffeine co-administration.

Locomotor activity was re-assessed after the fifth challenge injection on day 29 in the MDMA pre-exposure study and after the sixth challenge injection on day 16 in the caffeine study. In the MDMA pre-exposure study, MDMA and mephedrone caused sustained hyperactivity resulting in locomotor sensitisation to mephedrone in both vehicle and MDMA pre-exposed groups. Since both mephedrone groups developed an enhanced LMA response on day 29, and there was no difference between the groups on day 15, this apparent sensitisation occurred independently of MDMA pre-exposure, suggesting that the two compounds may induce hyperactivity via slightly different effects on dopamine and 5-HT release. The lack of sensitisation to MDMA in MDMA pre-exposed rats may be because these rats had already been exposed and sensitised to MDMA during the pre-exposure phase. In the caffeine co-administration study, mephedrone alone and in combination with caffeine also caused significant hyperactivity on day 16 (sixth injection). However, there was no sensitisation to the locomotor stimulating effects of mephedrone. This is

unexpected as the data presented in chapter 3, which followed a similar dosing schedule as the current study, showed sensitisation to mephedrone.

Finally PPI was investigated as an index of sensorimotor gating, which is attenuated by drugs that release 5-HT. MDMA has been reported to attenuate PPI in rats (Vollenweider et al. 1999). However the data presented in chapter 3 showed that five previous injections of chronic intermittent mephedrone (1-10 mg kg^{-1}) had no effect on PPI in the rat. In the current studies, after the sixth or fifth injections, there was again no significant effect of mephedrone alone, nor any combined effect following MDMA pre-exposure or caffeine co-administration.

Considering the evidence for locomotor sensitisation in the MDMA pre-exposure study, it is surprising that there were few changes in brain regional levels of 5-HT, dopamine and their major metabolites 60 min after the last challenge injection (after PPI). Although MDMA failed to decrease hippocampal 5-HT in the vehicle pre-exposed rats it decreased both 5-HT and 5-HIAA after MDMA pre-exposure (further evidence for the development of sensitisation in these rats), presumably due to MDMA-induced release of 5-HT from nerve endings, which results in an acute 5-HT depletion (Colado and Green 1994). This suggests that previous exposure to MDMA enhanced the 5-HT releasing effects of acute MDMA. In vehicle pre-exposed rats MDMA only decreased striatal 5-HIAA. Mephedrone also decreased hippocampal 5-HIAA in vehicle pre-exposed rats, probably due to acute neuronal 5-HT release (Kehr et al. 2011; Baumann et al. 2012; Motbey et al. 2012; Wright et al. 2012). However, this effect of mephedrone was absent in MDMA pre-exposed rats consistent with the proposal that cross-tolerance to the acute effects of MDMA and mephedrone on 5-HT release may develop. Importantly no significant 5-HT depletion occurred in rats previously exposed to MDMA and subsequently given vehicle, showing that repeated MDMA did not produce any 5-HT neurotoxicity.

There was no significant alteration in dopamine, 5-HT or their metabolites in any brain region dissected seven days after the last of six injections of

mephedrone alone or in combination with caffeine. Caffeine alone however did cause an increase in striatal dopamine, which may reflect a change in dopamine release and metabolism caused by caffeine-induced adenosine receptor activation.

Collectively, these findings suggest that previous recreational MDMA use or co-administration of caffeine may exacerbate some of the adverse effects of mephedrone, such as increasing hyperactivity and hyperthermia, while attenuating 'anxiety-like' behaviours. However the lack of complete cross-sensitisation to MDMA suggests that the sub-acute effects of MDMA and mephedrone may involve differences in their profile of activation of monoaminergic function (Eshleman et al. 2013).

Chapter 5 Effect of repeated mephedrone on core temperature and *in vivo* monoamine release

5.1 Introduction

MDMA users report the development of tolerance and often ingest repeated low doses within a short period of time to prolong its subjective effects, but dose escalation and binge use also increase the risk of adverse effects (Parrott 2005). Consistent with this, MDMA-induced increases in extracellular 5-HT are attenuated following binge-type administration in the rat (Rodsiri et al. 2011) whereas hyperthermia and hyperactivity are exacerbated (Green et al. 2004; Baumann et al. 2008; Rodsiri et al. 2011). Multiple re-dosing is also common with mephedrone users attempting to maintain the desired effects of this short-acting drug, and while a typical recreational dose is approximately 100 mg, individuals may take up to 4 g in a single binge session (Schifano et al. 2011; Winstock et al. 2011).

From the studies presented in Chapters 2 and 4, and previously published studies from other groups, the predominant thermoregulatory effect of mephedrone following acute administration in the rat appears to be hypothermia (Miller et al. 2013) even under conditions of elevated ambient temperature (Wright et al. 2012). However, hyperthermia has been observed in two studies following rapid repeated dosing (Hadlock et al. 2011; Baumann et al. 2012). Given the established association of hyperthermia with life threatening adverse effects of MDMA (Docherty and Green 2010), it is essential to see if there might be similar adverse risk with repeated mephedrone. The studies presented in the current chapter therefore examined the temporal profile of the temperature and locomotor response to short-term repeated mephedrone and established the involvement of serotonergic and dopaminergic neurons in these changes because of their established role in the effects of MDMA.

In the studies presented in this chapter, rats received three injections of mephedrone (10 mg kg^{-1}), at 2 h intervals to mimic the rapid re-dosing patterns typically favoured by many recreational users and to match previous preclinical 'binge-dosing' studies with MDMA and mephedrone (Baumann et al. 2008; Rodsiri et al. 2011; Baumann et al. 2012). Locomotor activity and core body temperature were monitored continually

using radiotelemetry to accurately and repeatedly record core body temperature without the stress associated with more invasive temperature monitoring techniques such as a rectal probe, which can cause confounding hyperthermia (Kramer and Kinter 2003). Since the predominant behavioural effect of mephedrone in previous studies was hyperlocomotion (Chapters 3 and 4) and the striatum plays a role in motor activity, extracellular dopamine efflux from this region was measured by *in vivo* microdialysis in the conscious rat to correlate neurotransmitter release with the observed behavioural effects. Previous chapters have shown that a 10 mg kg^{-1} dose of mephedrone produces robust physiological and behavioural changes in the rat and, importantly, pilot studies with up to 30 mg kg^{-1} of mephedrone confirm that the chosen 10 mg kg^{-1} dose produces sub-maximal changes in temperature and locomotion (Chapter 4), thereby allowing potential detection of either sensitisation or tolerance following repeated administration without concern for ceiling effects.

As previous studies suggest a role of dopamine in mephedrone-induced hypothermia (Chapter 2), the contribution of serotonergic and dopaminergic neurons to the physiological and behavioural effects of mephedrone were also examined using selective monoamine neurotoxins. Rats received bilateral intracerebroventricular (i.c.v.) injections of either 5,7-dihydroxytryptamine (5,7-DHT) or 6-hydroxydopamine (6-OHDA), to deplete 5-HT or dopamine respectively, and radiotelemetry was used to assess the impact of monoamine depletion on the locomotor stimulant and temperature effects of repeated mephedrone injection.

Following identification of a key role of 5-HT in mephedrone-induced temperature change, a final study investigated the specific 5-HT receptors mediating this effect by assessing the impact of selective 5-HT_{1A} and 5-HT_7 receptor antagonists on mephedrone-induced changes in rectal temperature. These two receptors were selected because of their known role in thermoregulation in the rat (Filip and Bader 2009; Gellynck et al. 2013). This is the first study to concomitantly examine the effects of repeated mephedrone injection on hyperactivity and hypothermia, as well as striatal dopamine efflux, in short time periods (20 min intervals to provide a good temporal resolution) and to establish the differential role of

dopamine and 5-HT in mephedrone-induced hyperactivity and hyperthermia to permit a comparison with the established effects of repeated MDMA injection.

5.2 Aims

The aims of this experiment were to:

(1) investigate of repeated 'binge-style' mephedrone administration on body temperature, locomotor activity and *in vivo* extracellular striatal dopamine efflux in the rat;

(2) identify the roles of 5-HT and dopamine in mephedrone-induced changes in body temperature and locomotor activity following repeated dosing;

(3) identify long term *ex vivo* neurochemical changes following repeated mephedrone injection and,

(4) to evaluate the role of serotonergic 5-HT_{1A} and 5-HT_7 receptors in mephedrone-induced hypothermia.

5.3 Materials and Methods

5.3.1 Animals

Experimentally naïve young-adult male Lister hooded rats (190-300g; Charles River UK) were used in all experiments. Rats were housed in groups of four prior to surgery and in individual wire-top cages post-surgery, under constant environmental conditions (12 h light:dark cycle with lights on at 07.00 h, ambient temperature 21 ± 2°C and relative humidity 55 ± 10%). Food and water were freely available and wet mash was provided for five days post-surgery. All experiments were conducted during the light phase between 09.00 h and 16.00 h. The dose and behavioural schedule used was chosen to comply with the three R's of

humane animal testing. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and ARRIVE guidelines with approval of University of Nottingham Local Ethical Committee.

5.3.2 Drugs

(±)-Mephedrone-HCl was purchased from Ascent Scientific, UK. Desipramine hydrochloride, ascorbic acid and 6-hydroxydopamine hydrobromide (6-OHDA) were purchased from Tocris Bioscience, UK. 5,7-dihydroxytryptamine creatine sulphate (5,7-DHT) was purchased from Sigma Aldrich, UK. Mephedrone and desipramine were dissolved in 0.154 M saline, and 6-OHDA and 5,7-DHT were dissolved in 0.2% w/v ascorbic acid. All doses are quoted as the salt.

5.3.3 Study 1: Effect of repeated mephedrone on locomotor activity and core body temperature

5.3.3.1 Radiotelemetry

Radiotelemetry allows for remote monitoring of behavioural and physiological changes in freely moving animals and was used in the current study as it allows for continuous, simultaneous monitoring of temperature and activity without the need for any undue stress to the animal that may be caused by conventional techniques, such as rectal temperature monitoring. The system used in this study (DataScience International, USA) uses a small implantable transmitter (Model TA10TA-F20) to record locomotor activity and core body temperature and relays them to the RPC-1 receivers. Data are then forwarded to the computerised A.R.T. v4 acquisition software via the data exchange matrix. Additionally, the handling required for rectal temperature monitoring would also prevent activity measurement in the same animal by infrared activity as measured in Chapters 3 and 4.

5.3.3.2 Implantation of radiotemetry transmitter

Sterile radio-transmitters (Model TA10TA-F20, DataScience International, USA) were surgically implanted into the peritoneal cavity of rats under anaesthesia, which was induced by inhalation of 4% isoflurane in N_2O:O_2 2:1 and maintained at 2% isoflurane during the procedure. Rats were placed on a heat pad during surgery to maintain body temperature. Local anaesthetic (EMLA cream, AstraZeneca, UK) was applied to the operative site and a small lateral incision of approximately 1.5 cm was made approximately 1 cm from the abdominal midline. The radiotransmitter was implanted vertically to minimise the likelihood of post-operative complications resulting from GI obstruction. The internal muscle layer was closed using individual absorbable sutures (Vicryl W9106, Ethicon, USA), and the skin closed using three wound clips (7 mm, Harvard Apparatus, USA). The wound was treated with anti-inflammatory gel (Fuciderm gel, Dechra, UK) and plastic wound dressing (Opsite, Smith and Nephew, UK). Post-operative fluids (1 ml saline) and analgesia (4 mg kg^{-1}, s.c. carprofen (Rimadyl), Pfizer, UK) were administered on the day of surgery and analgesia was continued for a further three days. Wound clips were removed seven days post-surgery and radiotelemetry recording commenced nine days post-surgery.

5.3.3.3 Data collection

Rats were transferred to the procedure room 24 h prior to testing. On the test day, core body temperature and activity were continuously monitored in the home cage at ambient room temperature (19.9-20.9 °C). Rats (n=5 per treatment group) received a total of three i.p. injections of either saline

vehicle (1 ml kg^{-1}) or mephedrone (10 mg kg^{-1}) at 2 h intervals. Data were collected for 10 s every 2 min starting 60 min prior (-60 min) to the first injection, grouped into 20 min epochs and expressed as mean ± SEM activity counts and change (°C) from baseline temperature readings obtained in the same individual (0 min). Total activity counts in the 120 min following each of the three injections were also calculated and are presented as mean ± SEM.

5.3.4 Study 2: Measurement of extracellular dopamine levels in the striatum by *in vivo* microdialysis following repeated mephedrone

5.3.4.1 Stereotaxic implantation of microdialysis guide cannulae

As the Animals (Scientific Procedures) Act, 1986 project licence in use at the time of these experiments would not allow radiotelemetry and microdialysis to be performed in the same animal, microdialysis was performed in a separate cohort of rats using a previously established technique (Rodsiri et al. 2011) with some modifications. A CMA 12 polyurethane guide cannula (CMA Microdialysis AB, Sweden) was implanted just above the striatum under general anaesthesia as described in section 5.3.3.2. In summary, rats were held in a stereotaxic frame by blunt ear bars (with EMLA cream at the tip) and an incisor bar set at -3.3 cm below the intra-aural line. A topical lubricant (Lacrilube, Allergan, UK) was applied to the eyes to prevent drying from the surgical lamps. Coordinates for the striatum were selected according to Paxinos and Watson (1997) Rat Brain Atlas (coordinates AP +0.48, ML ±3.0, DV -3.6 from Bregma). An incision was made between the eyes of the rat to the back of the head to allow for a small hole to be drilled into the skull above the coordinates using a microtrephan drill. The guide cannula was secured to the skull with three skull screws and dental cement (Associated Dental Products Ltd, UK). Guide cannulae were placed on alternating sides so that half of the rats in each treatment group had a microdialysis probe placed in the right striatum and half had a probe in the left striatum. Local analgesia

(Lidocaine hydrochloride) was applied before suturing the wound using interrupted sutures (Mersilk, W502, Ethicon, USA) and treated with plastic wound dressing and anti-inflammatory cream as in section 5.3.3.2. Post-operative fluids (1 ml saline) and analgesia (4 mg kg^{-1}, s.c. carprofen Rimadyl, Pfizer, UK) were administered on the day of surgery. Rats were housed individually and allowed to recover for eight days before dialysate collection commenced.

5.3.4.2 Microdialysis procedure

Seven days post-surgery rats were briefly anaesthetised to insert a microdialysis probe (CMA 12, 4 mm polyarylethersulpone membrane, 500 µm outer diameter, 3 µl internal volume with a 20 kDa molecular cut-off; CMA Microdialysis AB, Sweden) through the guide cannula. The probe was connected to a microinfusion pump (Harvard Scientific, USA) using FEP microdialysis tubing (Instech Laboratories Inc, USA) via a liquid swivel system (Instech 375/22, Instech Laboratories Inc, USA) to allow for unrestricted movement. Rats were placed in individual circular arenas (50 cm diameter, 45 cm height) with sawdust bedding, and food and water freely available. Artificial cerebrospinal fluid (125 mM NaCl, 13.5 mM $NaHCO_3$, 1.25 mM KCl, 0.22mM NaH_2PO_4, 0.9 mM Na_2HPO_4, 0.3 mM Na_2SO_4, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2 2H_2O$ adjusted to pH 7.4) was continuously perfused at a rate of 1 µl per min. the following day, rats (n=10 per treatment group) received a total of three i.p. injections at 2 h intervals of saline vehicle (1 ml kg^{-1}) or mephedrone (10 mg kg^{-1}) and dialysate samples were collected every 20 min into 5 µl of 0.1 M perchloric acid containing 0.03 % sodium metabisulfite. Samples were immediately stored on dry ice and then at -80 °C until analysis by HPLC-ED. The dead space from the probe to the collection tube resulted in a small delay in the dialysate samples reaching the collection point of approximately 3 min.

5.3.4.3 Histological confirmation of probe location

After collection of the final microdialysis sample, rats were euthanized by injection of 1 ml of pentobarbital (Euthatal). Brains were rapidly removed

and stored in 4 % paraformaldehyde solution at 4 °C until sectioning into 150 µm coronal slices using a vibrotome (Campden Instruments Ltd, UK). Location of the probe within the striatum was confirmed under a light microscope using Paxinos and Watson Rat Brain Atlas (1997). See Fig 5.1 for example placement of the microdialysis guide cannulae.

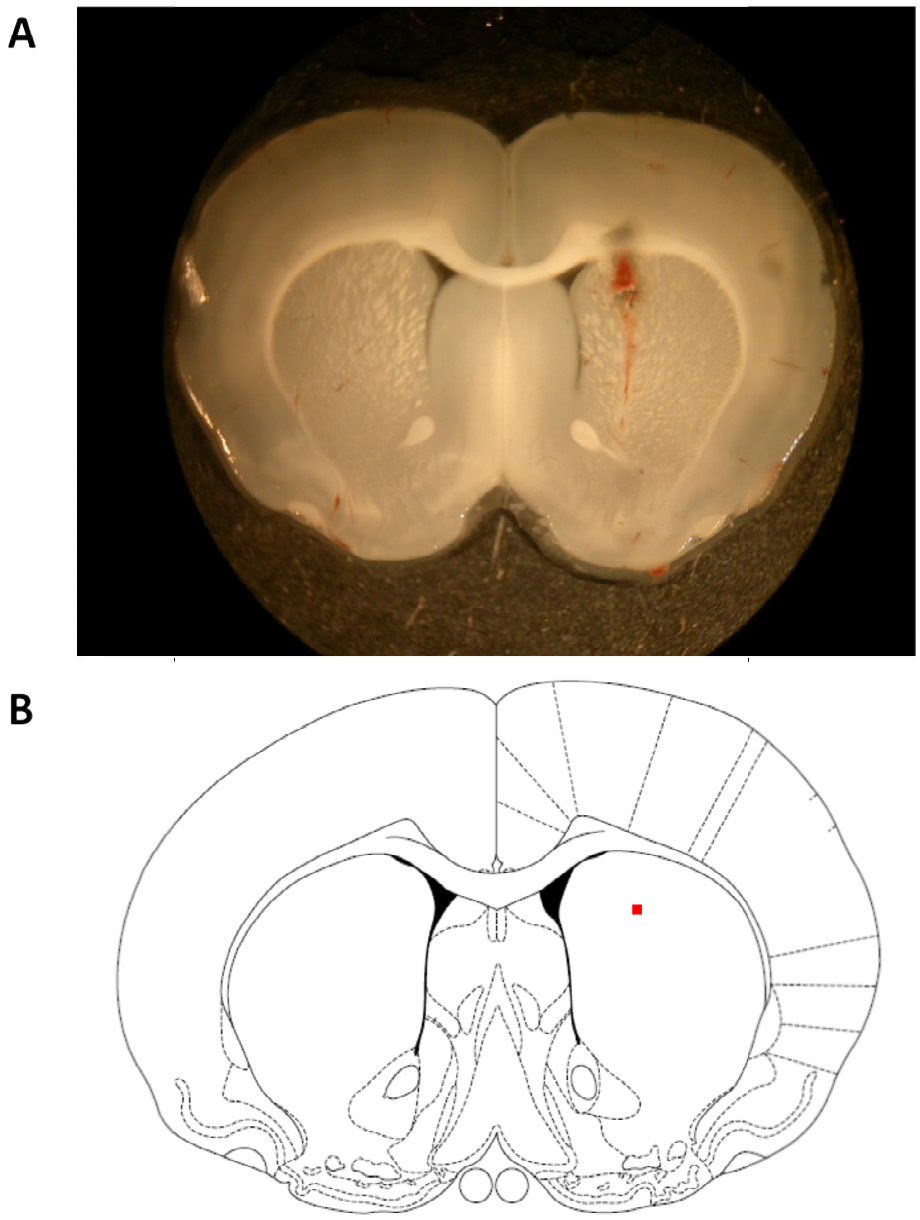


Figure 5.1 Histological confirmation of probe location within the striatum.

An example placement of the microdialysis guide cannula/probe in the striatum in (**A**) a fixed brain and (**B**) a diagram of the co-ordinates taken from Paxinos and Watson (1997) where the red dot marks the position of the guide cannulae. Microdialysis guide cannulae were placed on alternating sides so that half of the rats in each treatment group had a microdialysis probe placed in the right striatum and half had a probe in the left striatum.

5.3.5 Study 3: Effect of dopamine or 5-HT depletion on mephedrone-induced changes in core body temperature and activity

5.3.5.1 Intracerebroventricular injections

In a third group of rats, bilateral i.c.v. injections were performed under isoflurane anaesthesia as described in section 5.3.3.2. All rats received desipramine (15 mg kg^{-1}, i.p.) 30 min prior to i.c.v. injection to protect noradrenergic neurons from depletion by 5,7-DHT or 6-OHDA. Rats were held in a stereotaxic frame by blunt ear bars (with EMLA cream at the tip) and an incisor bar set at -3.3 cm below the intra-aural line. A topical lubricant (Lacrilube) was applied to the eyes to prevent drying from the surgical lamps. The stereotaxic coordinates for each injection site were AP -0.8, ML ±1.5, DV -3.8 from Bregma (Paxinos and Watson, 1997). An incision was made between the eyes of the rat to the back of the head to allow for a small hole was drilled into the skull above the coordinates using a microtrephan drill. Rats received 5 µl of 0.2 % w/v ascorbic acid vehicle, or 75 µg 5,7-DHT or 150 µg 6-OHDA into each lateral ventricle, at a rate of 5 µl min^{-1}. These doses were chosen as they are reported to deplete 5-HT and dopamine by a similar percentage (70-75 %) below control levels (Nowak et al. 2005; King et al. 2009). Local analgesia (Lidocaine hydrochloride) was applied before suturing the wound using interrupted sutures (Mersilk, W502, Ethicon, USA). The wound treated with plastic wound dressing and anti-inflammatory cream as in section 5.3.3.2.

5.3.5.2 Data collection by radiotelemetry

Radiotelemetry transmitters were also implanted into the same rats immediately after i.c.v. injection using the protocol described in section 5.3.3.2. Using a crossover design, each rat (n=8 per treatment group) received three i.p. injections at 2 h intervals of saline vehicle (1 ml kg^{-1}) or mephedrone (10 mg kg^{-1}) 21 days post-surgery, and then received the opposite treatment 28 days post-surgery. In each case core body temperature and activity were measured by radiotelemetry as described in

section 5.3.3.3. Thus every rat received both vehicle and mephedrone in a pseudorandom order to minimise inter-individual response to the drug treatment or lesion.

5.3.6 Study 4: Effect of 5-HT_{1A} and 5-HT_7 receptor antagonists on mephedrone-induced decreases in rectal temperature following a single injection

Rectal temperature measurements were recorded following combined 5-HT receptor antagonist and mephedrone administration to establish the specific 5-HT receptors involved in mediating mephedrone-induced hypothermia, using the method described in section 2.3.4. Rats (n=6 per treatment group) received saline vehicle (1ml kg^{-1}, i.p.), the 5-HT_{1A} antagonist WAY-100635 (0.5mg kg^{-1}) or the 5-HT_7 antagonist SB-258719 (10 mg kg^{-1}), followed 30 min later by vehicle (1ml kg^{-1}, i.p.) or mephedrone (10mg kg^{-1}). Rectal temperature was measured immediately prior to each injection and then at 20 min intervals for the next 2 h.

5.3.7 Neurochemistry

Seven days after radiotelemetry recording (d 35 following i.c.v. injections) rats were killed by concussion and immediate decapitation and hypothalamus and right striatum, frontal cortex and hippocampus were collected on a refrigerated table (4 °C), flash frozen in liquid nitrogen and stored at -80 °C until analysis of tissue dopamine, 5-HT and their major metabolites by HPLC-ED, as previously described (section 2.3.3.2). This HPLC-ED method was also used to quantify extracellular dopamine levels in microdialysis samples. In addition, tissue noradrenaline levels were measured in the same regions of sham, 5,7-DHT and 6-OHDA pre-treated rats by running the samples through the column a second time under modified HPLC conditions. The mobile phase for noradrenaline detection consisted of 20 mM KH_2PO_4/Na acetate, 8 mM KCl, 0.1 mM EDTA, 1 mM OSA, containing 10 % methanol, adjusted to pH 4.07.

5.3.8 Statistical analysis

All statistical analyses were performed using GraphPad Prism v 6.02 or SPSS v21 software. Radiotelemetry data were analysed by two-way repeated measures ANOVA (with drug treatment and time as between and within factors respectively) where the rats received saline vehicle or mephedrone alone (study 1), or by four-way repeated measures ANOVA (with i.c.v. injection and drug treatment as between-group factors and time and week as the within-group factors) where rats also received i.c.v. injections of ascorbic acid vehicle, 5,7-DHT or 6-OHDA and saline vehicle and mephedrone in a random order crossover design (study 3). Microdialysis data were analysed by two-way repeated measures ANOVA (with drug treatment and time as between and within factors respectively, study 2). HPLC data were analysed by one-way ANOVA where rats received saline vehicle or mephedrone alone (study 1) or by two-way ANOVA where they also received i.c.v. injections (study 3). Rectal temperature data were analysed by three-way repeated measure ANOVA (with 5-HT receptor antagonist and drug treatment as between factors and time as the within factor). Bonferroni multiple comparisons post-hoc test was used where appropriate and $p<0.05$ was considered statistically significant. All data are presented as mean ± SEM.

5.4 Results

5.4.1 Study 1: Effect of repeated mephedrone on core body temperature and activity

Mephedrone (3 x 10 mg kg^{-1} at 2 h intervals) was administered to a group of rats which had intraperitoneal radiotransmitter implants for recording of locomotor activity and body temperature by radiotelemetry.

5.4.1.1 Locomotor activity

Locomotor activity was recorded in the home cage of individually-housed rats to determine any 'psychostimulant-like' effect. There were no significant between-group differences in activity counts in the 60 min prior to the first injection (data not shown). Mephedrone significantly increased activity above vehicle control levels for 40 min after the first injection, and 80 min after the second and third injections, such that there were main effects of drug ($F_{(1,8)}$=15.5, p<0.01) and time ($F_{(18,144)}$=12.29, p<0.001), as well as a drug x time interaction ($F_{(18,144)}$=3.43, p<0.001, Fig 5.2). The magnitude of the increase produced by mephedrone was also similar after each injection although the response to saline injection appeared to be less with each consecutive injection.

Analysis of the three total cumulative activity counts in the 2 h following each consecutive injection confirmed that mephedrone caused a reproducible hyperactivity following each injection, such that there was no significant difference in total counts between injections (1^{st}: 580 ± 56; 2^{nd}: 567 ± 98; 3^{rd}: 416 ± 115 counts/2 h, mean ± SEM). However, in vehicle treated rats the total activity counts decreased from 197 ± 105 counts/2 h following the first injection and 108 ± 26 counts/2 h following the second to become significantly lower after the third injection (61 ± 15 counts/2 h; p<0.05 versus first injection) suggesting some habituation to the injection procedure. This was reflected by main effects of drug ($F_{(1,8)}$=16.44, p<0.01), injection number ($F_{(2,16)}$=15.34, p<0.001) and a drug x injection number interaction ($F_{(2,16)}$=3.87, p<0.05) which was therefore due to a change in the saline group rather than the mephedrone response.

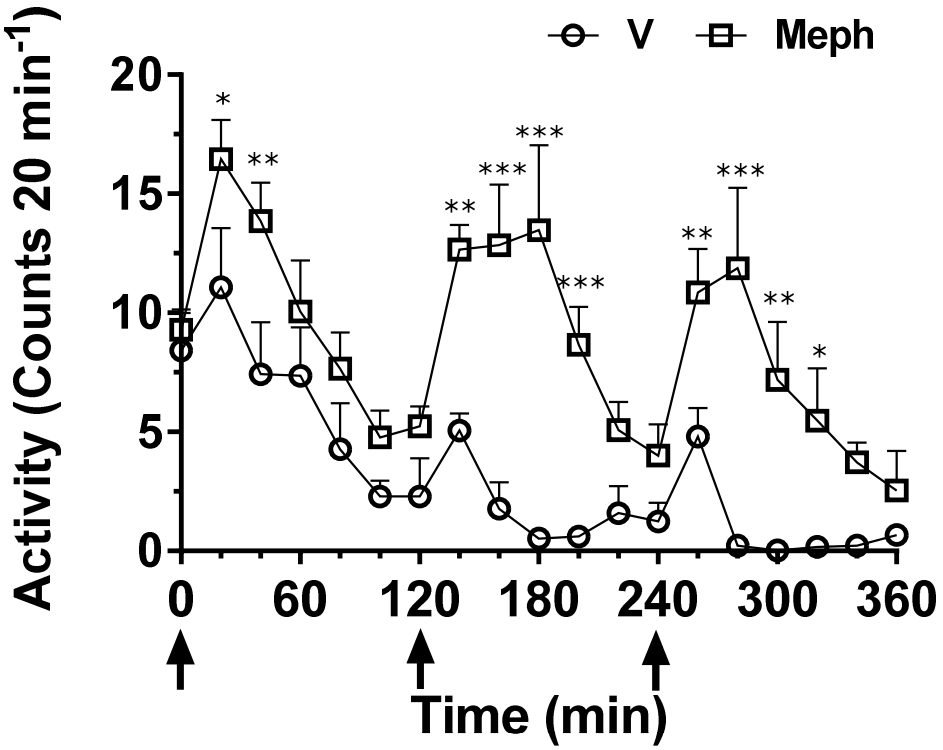


Figure 5.2 Mephedrone-induced hyperactivity is unaffected by binge administration of mephedrone.

Adult male Lister hooded rats (n=5 per treatment group) received i.p. injection of saline vehicle (V, 1 ml kg^{-1}) or mephedrone (Meph, 10 mg kg^{-1}) once every two hours at 0, 120 and 240 min (as indicated by the arrows). Locomotor activity was recorded continuously using a previously implanted radiotelemetry transmitter. Data are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle at the same time point, Bonferroni multiple comparisons post-hoc following two-way repeated measures ANOVA.

5.4.1.2 Core body temperature

Individual core body temperature was recorded simultaneously with activity in the home cage. There were no between-group differences in temperature in the 60 min prior to injection (data not shown), with baseline values (at the time of the first injection, t=0 min) being 37.8 ± 0.2 °C in rats allocated to vehicle and 37.9 ± 0.1 °C in those due to receive mephedrone. Following injection there were main effects of drug ($F_{(1,8)}$=3.27, p<0.05), time ($F_{(18,144)}$=14.67, p<0.001) as well as a drug x time interaction ($F_{(18,144)}$=4.26, p<0.001) although mephedrone decreased body temperature to a greater extent than vehicle control from 40-60 min after the first injection only (Fig 5.3).

The maximum temperature change from baseline following each mephedrone injection was similar being -1.3, -1.4 and -1.2 °C following the first, second and third injections respectively, yet this maximum change (like the time course data) was also only significantly different from the temperature change in the vehicle control group following the first injection. This appears to be because body temperature also decreased following the first vehicle injection and did not return to basal (pre-injection) values (Fig 5.3).

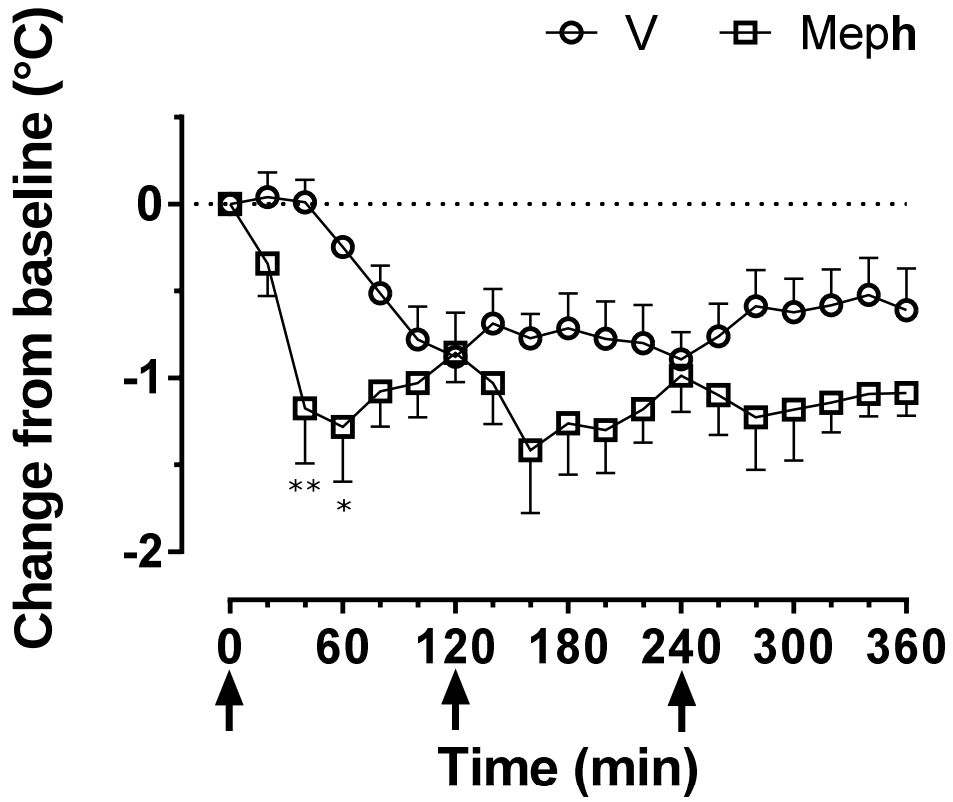


Figure 5.3 Repeated mephedrone injection does not alter mephedrone-induced changes in core body temperature.

Adult male Lister hooded rats (n=5 per treatment group) received i.p. injection of saline vehicle (V, 1 ml kg^{-1}) or mephedrone (Meph, 10 mg kg^{-1}) once every two hours at 0, 120 and 240 min (as indicated by the arrows). Core body temperature was recorded continuously via a previously implanted radiotelemetry transmitter. Data are presented as mean ± SEM change from baseline (t=0 min, °C). *p<0.05, **p<0.01, ***p<0.001 compared to vehicle, Bonferroni multiple comparisons post-hoc following two-way repeated measures ANOVA.

5.4.1.3 Ex vivo neurochemistry

Hypothalamus and right striatum, hippocampus and frontal cortex were collected seven days post-injection from rats implanted with radiotelemetry transmitters. There was no significant effect of mephedrone on the concentration of dopamine, 5-HT or their metabolites in any region (Table 5.1).

Table 5.1 Effect of repeated mephedrone administration on brain tissue dopamine and 5-HT levels seven days post-injection.

	Tissue levels (pmol mg^{-1})			
Treatment	Frontal cortex	Hippocampus	Hypothalamus	Striatum
	Dopamine			
V	0.25 ± 0.02	0.37 ± 0.3	4.64 ± 0.6	62.12 ± 9.4
Meph	0.19 ± 0.03	0.09 ± 0.01	2.89 ± 0.8	57.62 ± 15.4
	5-HT			
V	2.28 ± 0.2	1.78 ± 0.3	7.32 ± 1.2	4.15 ± 1.2
Meph	2.11 ± 0.3	1.37 ± 0.4	5.24 ± 1.2	6.31 ± 3.1

Dopamine and 5-HT levels were measured seven days after three i.p. injections of saline vehicle (V, 1 ml kg^{-1}) or mephedrone (Meph, 10 mg kg^{-1}) at 2 h intervals to individually-housed male Lister hooded rats (n=5 per treatment group) previously implanted with radiotelemetry transmitters under isoflurane anaesthesia. Data are expressed as mean ± SEM.

5.4.2 Study 2: Measurement of extracellular dopamine levels in the striatum by *in vivo* microdialysis

Extracellular striatal dopamine levels were measured by *in vivo* microdialysis in a separate cohort of rats. There were no between-group differences in basal extracellular dopamine levels in the 60 min prior to the first injection (7.32 ± 1.65 pmol ml^{-1} in rats due to receive vehicle and 5.08 ± 0.85 pmol ml^{-1} in those to receive mephedrone). Following injection, ANOVA showed a main effect of drug ($F_{(1,18)}$=6.29, $p<0.05$), time ($F_{(18,319)}$=7.87, $p<0.001$) as well as a drug x time interaction ($F_{(18,319)}$=3.55, $p<0.001$, Fig 5.4) and mephedrone rapidly increased extracellular striatal dopamine levels above vehicle controls for 40 min after the first and third injections and for 60 min after the second injection, with a return to baseline between injections.

Peak dopamine levels in mephedrone-treated rats were 284, 521 and 435% above basal following the first, second and third injections, respectively which were all significantly greater than that seen with vehicle treatment ($p<0.01$ to $p<0.001$) at the same time point. Thus the three consecutive injections of mephedrone each produced a comparable magnitude and equivalent time course of elevation in extracellular dopamine overflow in the striatum.

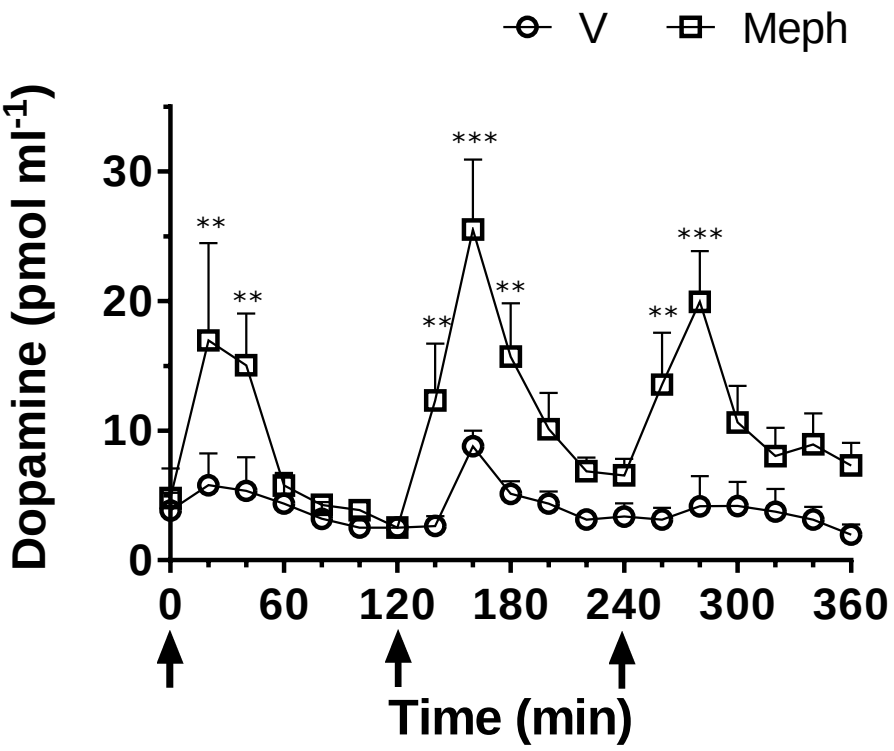


Figure 5.4 Mephedrone injection increases extracellular striatal dopamine efflux as measured by in vivo microdialysis.

Adult male Lister hooded rats (n=10 per treatment group) received i.p. injection of saline vehicle (V, 1 ml kg^{-1}) or mephedrone (Meph, 10 mg kg^{-1}) once every two hours at 0, 120 and 240 min (as indicated by the arrows). Data are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle, Bonferroni multiple comparisons post-hoc following two-way repeated measures ANOVA.

5.4.3 Study 3: Effect of dopamine or 5-HT depletion on mephedrone-induced changes in activity and core body temperature

A third group of rats received bilateral i.c.v. injections of 5,7-DHT or 6-OHDA to investigate the contribution of 5-HT and dopamine to mephedrone-induced hyperactivity and hypothermia.

5.4.3.1 Locomotor activity

There were no between-group differences in basal activity recorded in the 60 min prior to the first drug injection and, consistent with the previous experiment, mephedrone caused a rapid increase in locomotor activity which returned to basal levels between each injection whereas vehicle produced a very transient and small response in the same rats (Fig 5.5). Four-way repeated measures ANOVA confirmed main effects of drug ($F_{(1,18)}$=237.25, $p<0.001$), lesion ($F_{(2,18)}$=22.3, $p<0.001$) and time ($F_{(18,342)}$=28.51, $p<0.001$) as well as drug x lesion ($F_{(2,42)}$=12.87, $p<0.001$) and drug x time ($F_{(18,342)}$=13.27, $p<0.001$) interactions, but no drug x lesion x time interaction ($F_{(36,342)}$=0.96, $p>0.05$). Of note, 5,7-DHT pre-treatment attenuated the mephedrone-induced hyperactivity such that there was no significant response to the first injection in 5,7-DHT treated rats and the responses to the second and third injections were significantly lower in 5-,7-SHT pre-treated rats than sham controls (Fig 5.5A). In contrast, 6-OHDA lesion appeared to enhance the locomotor activity induced by mephedrone compared to that observed in sham controls, although this effect only reached significance at 40 min after the second injection (Fig 5.5B).

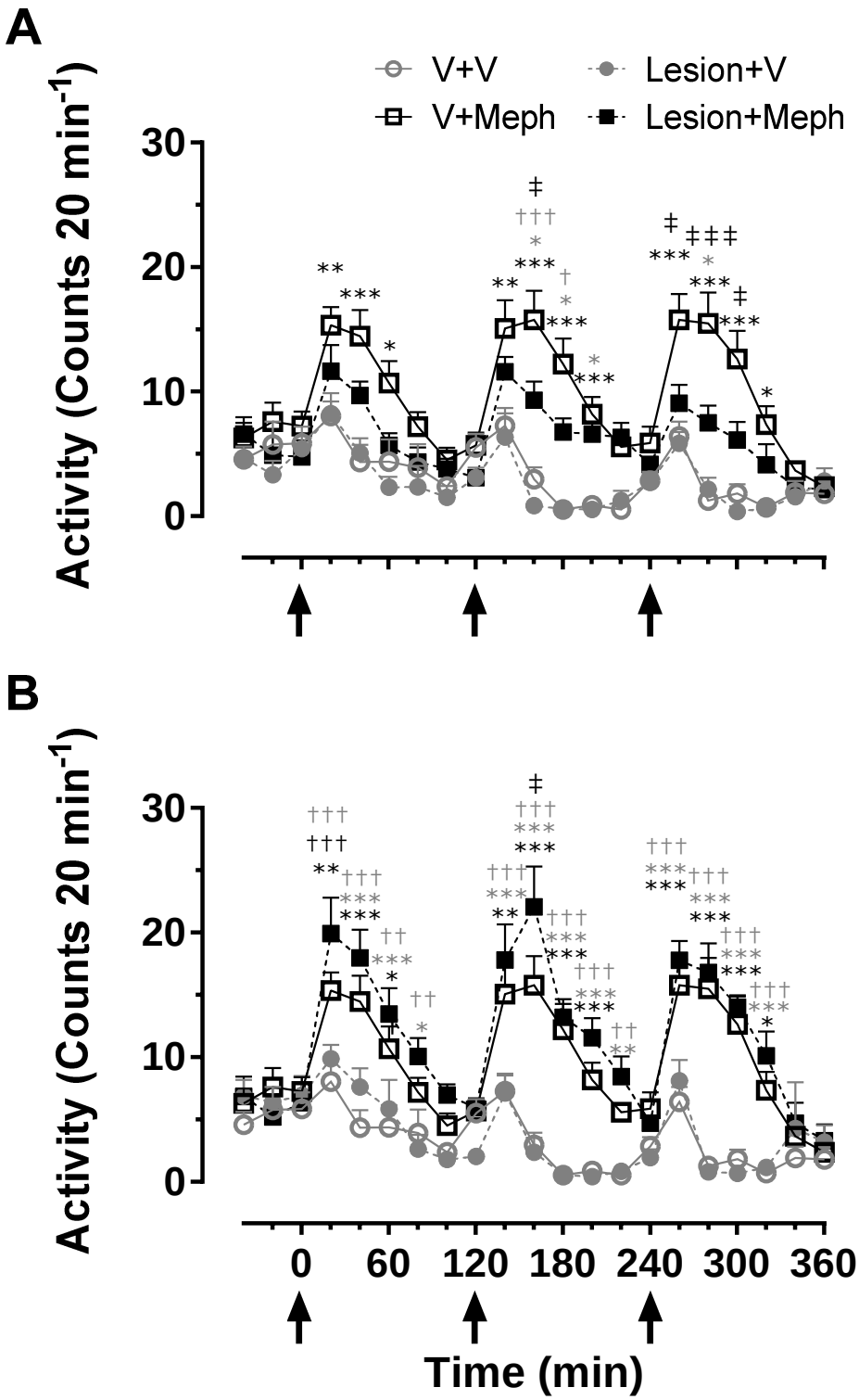


Figure 5.5 5-HT and dopamine depletions have different effects on mephedrone-induced changes in locomotor activity.

Adult male Lister hooded rats (n=8 per treatment group) that had radiotelemetry transmitters implanted under isoflurane anaesthesia received i.c.v. injection of 0.2 % ascorbic acid vehicle (5 µl), (**A**) 5,7-DHT (75 µl) or (**B**) 6-OHDA (150 µl), under

isoflurane anaesthesia, followed by repeated i.p. injection of saline vehicle (1 ml kg^{-1}) or mephedrone (10 mg kg^{-1}) on day 21 post-surgery and then the opposite treatment 28 days post-surgery. Vehicle and mephedrone were injected once every two hours at 0, 120 and 240 min (as indicated by the arrows). For clarity 5,7-DHT and 6-OHDA have been presented as separate figures versus the sham controls but ANOVA has been performed on all groups. All data are represented as mean ± SEM, *p<0.05, ** p<0.01, ***p<0.001 sham + mephedrone compared to sham + vehicle ; ***p< 0.001, **p< 0.01, *p< 0.05 lesion + mephedrone compared to sham + vehicle, †††p<0.001, ††p<0.01, †p<0.05 lesion + mephedrone compared to lesion + vehicle, ‡‡‡p<0.001, ‡p<0.05 lesion + mephedrone compared to sham + mephedrone, Bonferroni multiple comparisons post-hoc following two-way repeated measures ANOVA.

Consistent with the previous experiment, analysis of the total cumulative activity counts in the 2 h following each injection confirmed mephedrone-induced hyperactivity in sham operated controls and ANOVA showed main effects of drug ($F_{(1,18)}$=248.18, p<0.001), injection number ($F_{(2,36)}$=4.76, p<0.05) and a drug x injection number interaction ($F_{(2,36)}$=5.31, p<0.01, Table 5.2). Lesions significantly altered total mephedrone-induced activity counts such that there was a main effect of lesion ($F_{(2,18)}$=22.06, p<0.001) and a drug x lesion interaction ($F_{(2,18)}$=13.29, p<0.001). Consistent with the time course data, 5,7-DHT attenuated the response to mephedrone, such that there was a significant response to the second injection only (p<0.001 versus vehicle) and total activity counts following the third mephedrone injection were lower (p<0.01) in 5,7-DHT lesioned rats than sham operated controls. In contrast, 6-OHDA lesioned rats exhibited increased cumulative activity following each mephedrone injection which did not differ from the response in sham controls following any of the three injections.

Table 5.2 Total activity counts following repeated mephedrone administration to 5-HT or dopamine depleted rats.

Lesion	Treatment	Injection 1	Injection 2	Injection 3
Sham	V	285 ± 63	149 ± 25	139 ± 23†
	Meph	579 ± 77**	619 ± 70***	572 ± 73***
5,7-DHT	V	226 ± 40	123 ± 23	134 ± 26
	Meph	382 ± 44	444 ± 43**†††	313 ± 50‡‡
6-OHDA	V	298 ± 50	132 ± 20†	182 ± 57
	Meph	743 ± 63***†	766 ± 72***†††	667 ± 39***†††

Activity counts were measured following each of three i.p. injections of saline vehicle (V, 1 ml kg^{-1}) or mephedrone (Meph, 10 mg kg^{-1}) at 2 h intervals, 21 or 28 days after pre-treatment with bilateral i.c.v. injection under isoflurane anaesthesia of 0.2 % ascorbic acid vehicle (5 µl), 5,7-DHT (75 µg) or 6-OHDA (150 µg) per side, to individually-housed adult male Lister hooded rats (n=8 per treatment group). **p<0.01, ***p<0.001 sham + mephedrone compared to sham + vehicle; **p<0.01, ***p<0.001 lesion + mephedrone compared to sham + vehicle, †p<0.05, †††p<0.001 lesion + mephedrone compared to lesion + vehicle, ‡‡p<0.01 lesion + mephedrone compared to sham + mephedrone, †p<0.05 compared to the first injection of the same treatment group, Bonferroni multiple comparisons post-hoc following three-way repeated measures ANOVA.

5.4.3.2 Core body temperature

Basal core body temperatures prior to the first injection on each test day were equivalent bring 37.2 ± 0.2 and 37.5 ± 0.2 °C in sham controls, 36.9 ± 0.2 and 37.2 ± 0.2 °C following 5,7-DHT and 37.5 ± 0.2 and 37.4 ± 0.2 °C 6-OHDA treated rats prior to injection of vehicle or mephedrone respectively. There were main effects of drug ($F_{(1,18)}$=8.75, $p<0.01$), lesion ($F_{(2,18)}$=4.26, $p<0.05$) and time ($F_{(18,324)}$=12.55, $p<0.001$) as well as drug x time ($F_{(18,324)}$=9.19, $p<0.001$), lesion x time ($F_{(36,324)}$=1.78, $p<0.01$) and drug x lesion x time interactions ($F_{(36,324)}$=2.10, $p<0.001$, Fig 5.6). Mephedrone significantly reduced core body temperature in sham controls 20-80 min after the first, 20-60 min after the second and at 40 and 80 min following the third injection. However, mephedrone-induced hypothermia was completely abolished in 5,7-DHT lesioned rats, such that the temperature did not differ from that observed with vehicle at any time point, and was significantly attenuated compared to mephedrone-treated sham controls (Fig 5.6A). Although the duration of mephedrone-induced hypothermia appeared to be reduced by dopamine depletion in 6-OHDA pre-treated rats (such that the decrease in temperature was only significant from 40-80 min and at 40 min following the first and second injections, respectively, instead of the entire post-injection period), the magnitude of the mephedrone-induced hypothermia remained similar to that in sham controls (Figure 5.6B). The maximum temperature change from baseline following each mephedrone injection in sham controls (-1.2, -1.2 and -1.0 °C following that first, second and third injections, respectively) was equivalent.

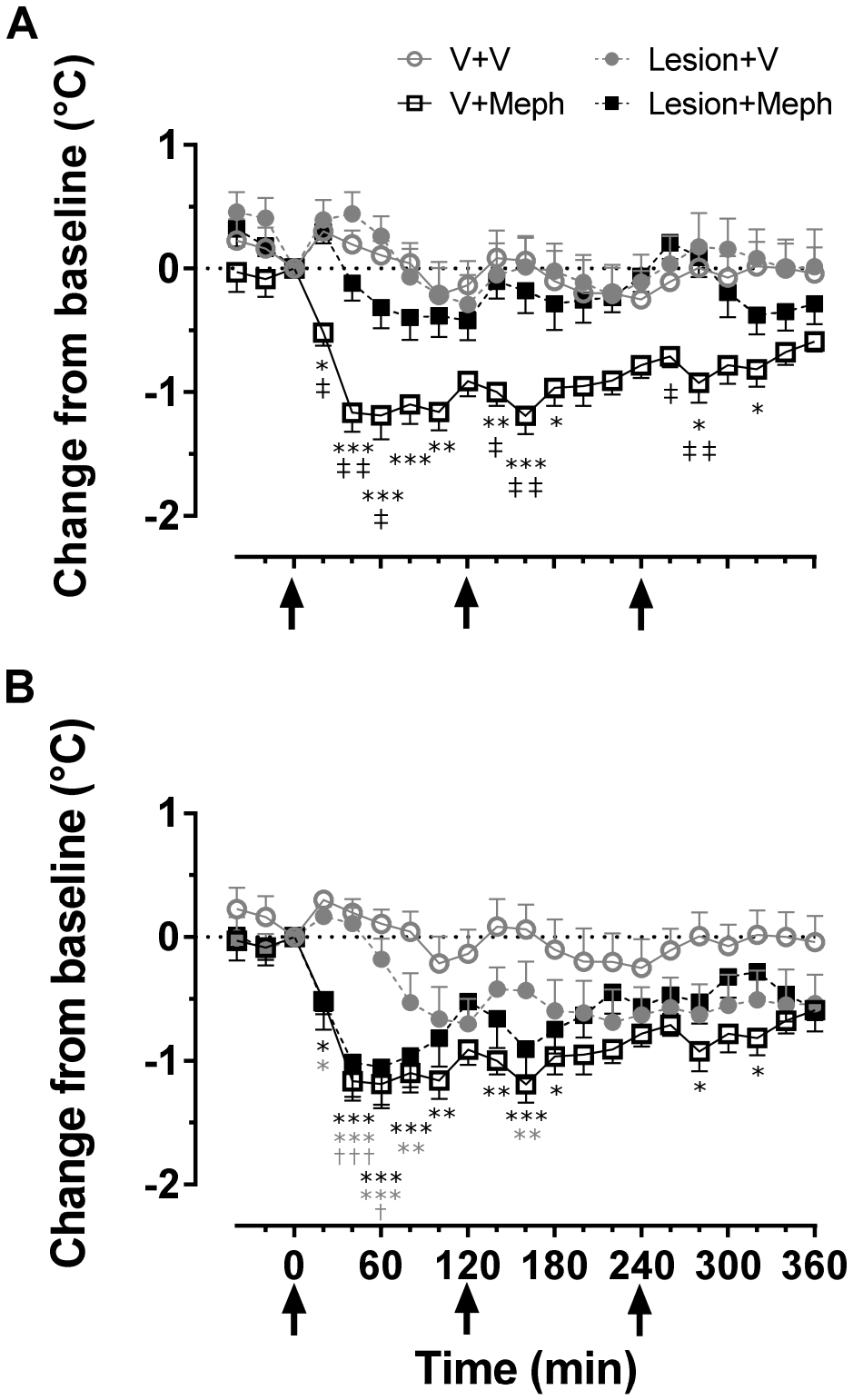


Figure 5.6 5-HT and dopamine depletion had different effects on mephedrone-induced hypothermia.

Adult male Lister hooded rats (n=8 per treatment group) that had radiotelemetry transmitters implanted under isoflurane anaesthesia received i.c.v. injection of 0.2

% ascorbic acid vehicle (5 µl), (**A**) 5,7-DHT (75 µl) or (**B**) 6-OHDA (150 µl) followed by repeated i.p. injection of saline vehicle (1 ml kg^{-1}) or mephedrone (10 mg kg^{-1}) on day 21 post-surgery and the opposite treatment on day 28 post-surgery using a cross over design. Vehicle and mephedrone were injected once every two hours at 0, 120 and 240 min (as indicated by the arrows). For clarity 5,7-DHT and 6-OHDA have been presented as separate figures versus the sham controls but ANOVA has been performed on all groups. Temperature data is represented as change from baseline (t=0 min, °C). All data are represented as mean ± SEM., *p<0.05, **p<0.01, ***p<0.001 sham + mephedrone compared to sham + vehicle; *p<0.05, **p<0.01, ***p<0.001 lesion + mephedrone compared to sham + vehicle; †p<0.05, †††p<0.001 lesion + mephedrone compared to lesion + vehicle; ‡p<0.05, ‡‡‡p<0.01 lesion + mephedrone compared to sham + mephedrone, Bonferroni multiple comparisons post-hoc following two-way repeated measures ANOVA.

5.4.3.3 Ex vivo neurochemistry

Dopamine, 5-HT and noradrenaline levels in the hypothalamus, right frontal cortex, hippocampus and striatum were measured 35 days after neurotoxin administration to confirm selective monoamine depletion. As expected the serotonergic neurotoxin, 5,7-DHT significantly reduced 5-HT to 46% of control in the frontal cortex ($F_{(2,21)}$=16.67, p<0.001), 13% in the hippocampus ($F_{(2,20)}$=7.19, p<0.01) and 42% in the hypothalamus ($F_{(2,21)}$=20.95, p<0.001) and 66% in the striatum, although the latter did not reach statistical significance due to high individual variation (Table 5.3). In contrast, 6-OHDA significantly reduced dopamine to 52% of control in the striatum ($F_{(2,21)}$=15.86, p<0.001) and 56%, 80% and 86% of control in the frontal cortex, hippocampus and hypothalamus, respectively, although the depletion in these areas was not statistically significant. However, the 6-OHDA-induced decrease in striatal dopamine was accompanied by a significant reduction in hippocampal 5-HT ($F_{(2,20)}$=7.19, p<0.01) as well as decreased noradrenaline levels in the hypothalamus and hippocampus (p<0.001), but noradrenaline levels were unchanged in the other regions examined.

Table 5.3 Effect of i.c.v. administration of 6-OHDA or 5,7-DHT on brain tissue dopamine, 5-HT and noradrenaline levels 5 weeks post-surgery.

	Tissue levels (pmol mg^{-1})			
Lesion	Frontal cortex	Hippocampus	Hypothalamus	Striatum
	Dopamine			
Sham	0.72 ± 0.2	0.40 ± 0.03	2.96 ± 0.2	55.58 ± 4.5
5,7-DHT	0.46 ± 0.02	0.38 ± 0.02	3.36 ± 0.2	60.42 ± 2.2
6-OHDA	0.40 ± 0.02	0.32 ± 0.01	2.53 ± 0.2	28.63 ± 5.5***
	5-HT			
Sham	3.46 ± 0.3	3.81 ± 0.4	7.22 ± 0.5	4.40 ± 0.3
5,7-DHT	1.58 ± 0.3***	0.51 ± 0.1***	3.03 ± 0.4***	2.88 ± 0.5
6-OHDA	3.96 ± 0.2	1.91 ± 0.6*	5.39 ± 0.6	5.50 ± 0.5
	Noradrenaline			
Sham	1.83 ± 0.08	2.81 ± 0.3	16.77 ± 1.5	0.93 ± 0.1
5,7-DHT	1.86 ± 0.1	2.21 ± 0.5	15.90 ± 1.5	1.12 ± 0.2
6-OHDA	1.70 ± 0.08	0.86 ± 0.2**	8.46 ± 1.4**	1.02 ± 0.3

Dopamine, 5-HT and noradrenaline levels were measured five days after bilateral i.c.v. injection under isoflurane anaesthesia of 0.2 % ascorbic acid vehicle (5 µl), 5,7-DHT (75 µg) or 6-OHDA (150 µg) per side to individually-housed male Lister hooded rats (n=8 per treatment group). Rats also received vehicle or mephedrone on day 21 post-surgery and then the opposite treatment on day 28 post-surgery, using a cross over design. *p<0.05, **p<0.01, ***p<0.001 compared to sham controls, Bonferroni post-hoc following one-way ANOVA.

5.4.4 Study 4: Effect of 5-HT_{1A} and 5-HT_7 receptor antagonists on mephedrone-induced decreases in rectal temperature following a single injection

In a final study, rats were pre-treated i.p. with either the selective 5-HT_{1A} receptor antagonist, WAY-100635 or the selective 5-HT_7 receptor antagonist, SB-258719 to investigate the role of specific 5-HT receptors in mephedrone-induced hypothermia. As before, basal temperature was identical in all groups prior to acute drug injection (data not shown). Subsequent injection of vehicle had no effect on rectal temperature irrespective of whether rats were pre-treated with WAY-100635 (Fig 5.7A) or SB-258719 (Fig 5.7B). Mephedrone caused a transient but significant decrease in rectal temperature at 20 ($p<0.001$) and 40 min ($p<0.05$) post-injection compared with vehicle control, which was consistent with the duration and magnitude observed in Chapter 2. This mephedrone-induced hypothermia was partially blocked by WAY-100635 (pre-treatment x drug x time interaction: $F_{(7,140)}=5.42$, $p<0.001$, being reduced in the 20-40 min post-mephedrone period, Fig 5.7A), but remained completely unaltered by SB-258719 (Fig 5.7B).

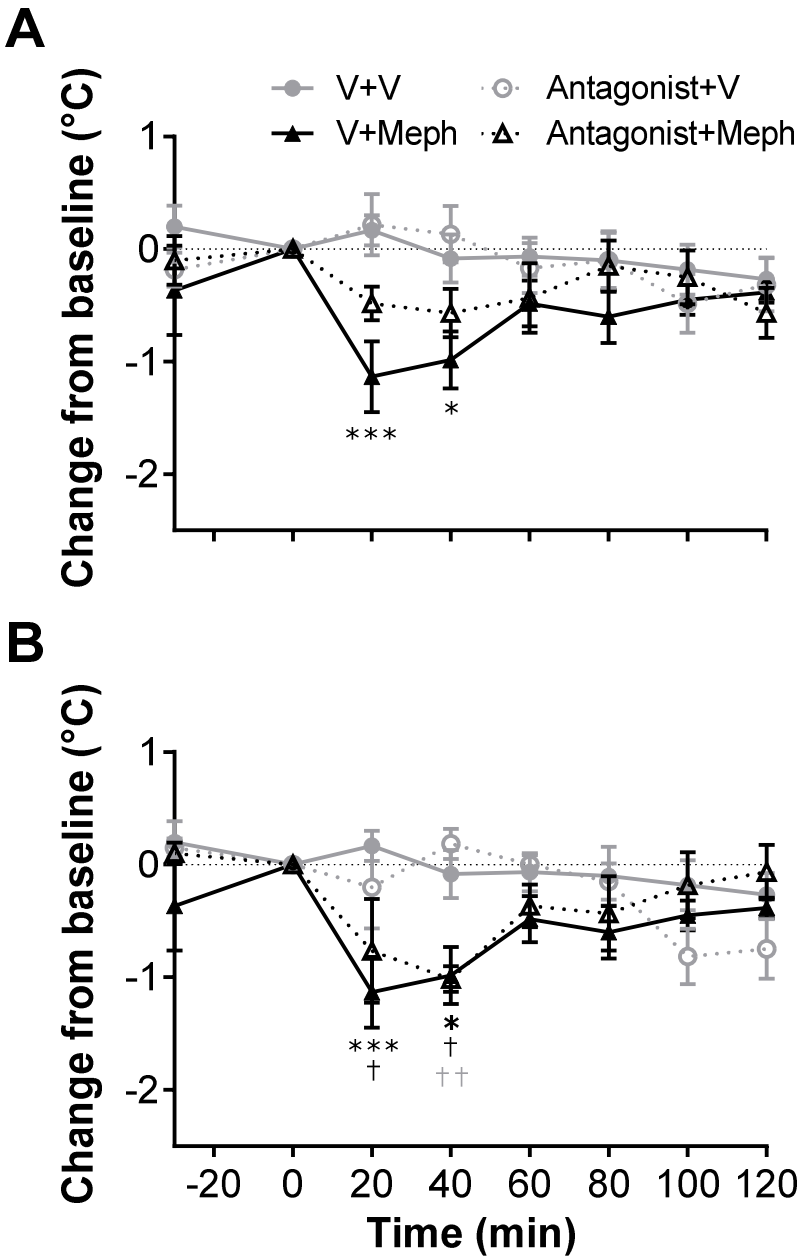


Figure 5.7 Comparison of the effect of the 5-HT1A receptor antagonist WAY-100635 and the 5-HT7 receptor antagonist SB-258719 on saline vehicle (1ml kg^{-1}) or mephedrone (10 mg kg^{-1}) induced change in rectal temperature.

Adult male Lister hooded rats (n=6 per treatment group) received i.p. saline vehicle (1 ml kg^{-1}), (**A**) WAY-100635 (0.5 mg kg^{-1}) or (**B**) SB-258719 (10mg kg-1) at -30 min, before saline (1 ml kg^{-1}) or mephedrone (10 mg kg^{-1}) at time=0 min. Rectal temperature was measured at -30 min and at 20 min intervals from 0 to 120 min, and data are expressed as change in temperature (°C, mean ± SEM) from the reading taken at 0 min. *p<0.05, ***p<0.001 vehicle mephedrone versus vehicle+vehicle; †p<0.05 antagonist+mephedrone versus vehicle+vehicle;

††p<0.05 antagonist+mephedrone versus antagonist+vehicle, Bonferroni post-hoc following three-way repeated measures ANOVA.

5.5 Discussion

This study investigated the effects of repeated mephedrone injection on core body temperature, locomotor activity and striatal dopamine release in the rat, since recreational users often rapidly re-dose, and examined the role of dopamine and 5-HT in mephedrone-induced changes in body temperature and activity. This is the first study to use radiotelemetry to obtain a high temporal resolution of changes appropriate for these short duration responses. The main findings were that (1) the hyperactivity, hypothermia and increase in extracellular striatal dopamine seen after the first of three mephedrone injections were comparable in magnitude and time course to those seen after the second and third injections, (2) dopamine depletion enhanced hyperactivity but reduced the duration of the hypothermic response to mephedrone, (3) 5-HT depletion reduced hyperactivity and abolished mephedrone-induced hypothermia and (4) 5-HT_{1A} receptor antagonism partially blocked the mephedrone-induced hypothermic response but 5-HT_7 receptor antagonism had no effect. Importantly, some of these observed effects contrast with those reported for MDMA, suggesting the possibility of adverse effects following recreational use.

Initial studies investigating the effects of mephedrone in rats have shown that it has a high affinity for dopamine and 5-HT transporters as well as VMAT-2, the 5-HT_{2A} and 5-HT_{2C} receptors, and α_{1A}- and α_{2A}-adrenoceptors (Lopez-Arnau et al. 2012; Martinez-Clemente et al. 2012; Eshleman et al. 2013; Simmler et al. 2013). Mephedrone also increases extracellular dopamine and to an even greater extent 5-HT in the nucleus accumbens (Kehr et al. 2011; Baumann et al. 2012; Wright et al. 2012; Eshleman et al. 2013). The current study shows that mephedrone also causes a short-lasting increase in extracellular striatal dopamine in the striatum, the temporal profile of which was similar following each injection and returned to baseline before the next injection. The repeated dose of mephedrone herein given (10 mg kg^{-1}) did not produce any neurotoxic loss of brain regional dopamine or 5-HT measured seven days post-injection. This is in marked contrast to MDMA, where a repeated dose schedule which releases striatal dopamine also produces significant long-term neurotoxic 5-HT

depletion in the rodent (Green et al. 2003), but similar to methcathinone where a much larger dose than that needed to elicit behavioural changes is required to obtain neurotoxicity (Sparago et al. 1996). These data therefore suggest that rapid repeated mephedrone administration is less likely to produce monoamine neurotoxicity than MDMA in rats, and supports the findings of Baumann et al, (2012). However, repeated high dose administration to mice does cause both dopaminergic and serotonergic neurotoxicity (Martinez-Clemente et al. 2014). It is important to note that while dopamine alterations within the striatum was measured in this study, the hypothalamus is more likely to be responsible for the temperature changes and there may be regional differences in the effect of mephedrone on monoamine release.

There is extensive evidence that mephedrone induces hyperactivity in rodents following both acute and intermittent administration (Kehr et al. 2011; Angoa-Perez et al. 2012; Baumann et al. 2012; Marusich et al. 2012). In the current study, repeated 'binge-style' mephedrone administration caused reproducible hyperactivity after each injection, the onset of which occurred within minutes of injection but returned to baseline levels within an hour. The time courses for both striatal dopamine release and hypothermia are consistent with a previous study using a single systemic injection. It is noteworthy that the peak plasma level of mephedrone in the rat shows a similar temporal pattern following s.c. injection (Miller et al. 2013). Importantly, the total ambulatory activity counts following the second and third injections of mephedrone were comparable to those following the first injection. This response therefore differs markedly from MDMA where enhanced hyperactivity was observed following a similar repeated dosing schedule in the same strain of rats (Rodsiri et al. 2011).

In the current study, central 5-HT depletion markedly attenuated the hyperactivity observed following mephedrone injection 21 or 28 days later, while central depletion of dopamine enhanced mephedrone-induced hyperactivity. Administration of 300 mg kg^{-1} of pCPA to mice over three days also reduces mephedrone-induced hyperactivity (Lopez-Arnau et al. 2012). Previous studies show that dopamine denervation supersensitivity

can occur following 6-OHDA administration, enhancing the hyperlocomotor response to injection of a dopamine D_1 receptor antagonist (Bishop et al. 2003). Since striatal dopamine was only decreased by 52% in the current study a similar supersensitivity to mephedrone-induced dopamine release could account for the enhanced locomotor response in 6-OHDA lesioned rats. The current findings therefore suggest a key role for both dopamine and 5-HT in mephedrone-induced hyperactivity.

Although hyperthermia has not been recorded in mephedrone users there is evidence that it alters peripheral thermoregulation since reported adverse effects include cold/blue fingers, hot flushes and sweating (Winstock et al. 2011; Wood and Dargan 2012). These changes may result from peripheral changes in blood flow. Earlier studies have generally failed to observe hyperthermia in rodents given an acute injection of mephedrone, even when the animals are grouped or kept under conditions of raised ambient temperature (Wright et al. 2012). However, hyperthermia was observed in two previous studies investigating the effects of repeated mephedrone injection (Hadlock et al. 2011; Baumann et al. 2012). Of note, both of these used Sprague Dawley rats and s.c. injections so there could be strain and/or pharmacokinetic differences in the response (Wright et al. 2012). These repeated injection studies also used a rectal probe to measure the response at 1 h intervals so the observed hyperthermia may have resulted from an additive effect of repeated mephedrone injection combined with stress-induced hyperthermia associated with rectal temperature measurement as evident in vehicle control animals (Hadlock et al. 2011; Baumann et al. 2012). The current study is one of the first to use radiotelemetry to measure the temperature response following repeated mephedrone injection. Since this study observed hypothermia even when rapid repeat dosing was performed it seems unlikely that recreational mephedrone use will result in the severe and sometimes fatal hyperthermia seen in high dose MDMA users.

Evidence suggests that dopamine and noradrenaline are important for MDMA-induced temperature changes (Mechan et al. 2002; Bexis and Docherty 2005; Green et al. 2005; Bexis and Docherty 2009). In addition,

5-HT depletion by *p*-chlorophenylalanine or a neurotoxic dose of MDMA disrupts the ability of the rat to regulate its response to a subsequent dose of MDMA (Green et al. 2004; Saadat et al. 2005). Of particular note, in the current study, 5,7-DHT lesion of serotonergic neurons abolished the hypothermic response to mephedrone. Furthermore, administration of the 5-HT_{1A} receptor antagonist, WAY-100635, attenuated mephedrone-induced hypothermia while antagonism of the 5-HT_7 receptor had no effect. Although 5-HT_{1A} receptors are implicated in the hypothermic response their involvement is almost certainly a consequence of 5-HT release and/or inhibition of reuptake, since the low affinity of mephedrone for the 5-HT_{1A} receptor (Ki>20 μM; Simmler et al, 2013) makes any direct effect unlikely. Interestingly, pre-treatment with WAY-100635 also prevents the hypothermic response to MDMA (Rusyniak et al. 2007). In contrast, mephedrone injection to 6-OHDA lesioned rats produced a hypothermic response which did not differ from mephedrone-induced hypothermia seen in sham controls. At first this appears paradoxical since administration of the dopamine D_1 receptor antagonist, SCH 23390 prolonged mephedrone-induced hypothermia in a previous study (Chapter 2). However, the limited depletion of dopamine in the hypothalamus makes it difficult to come to any firm conclusion about the role of dopamine in mephedrone-induced hypothermia.

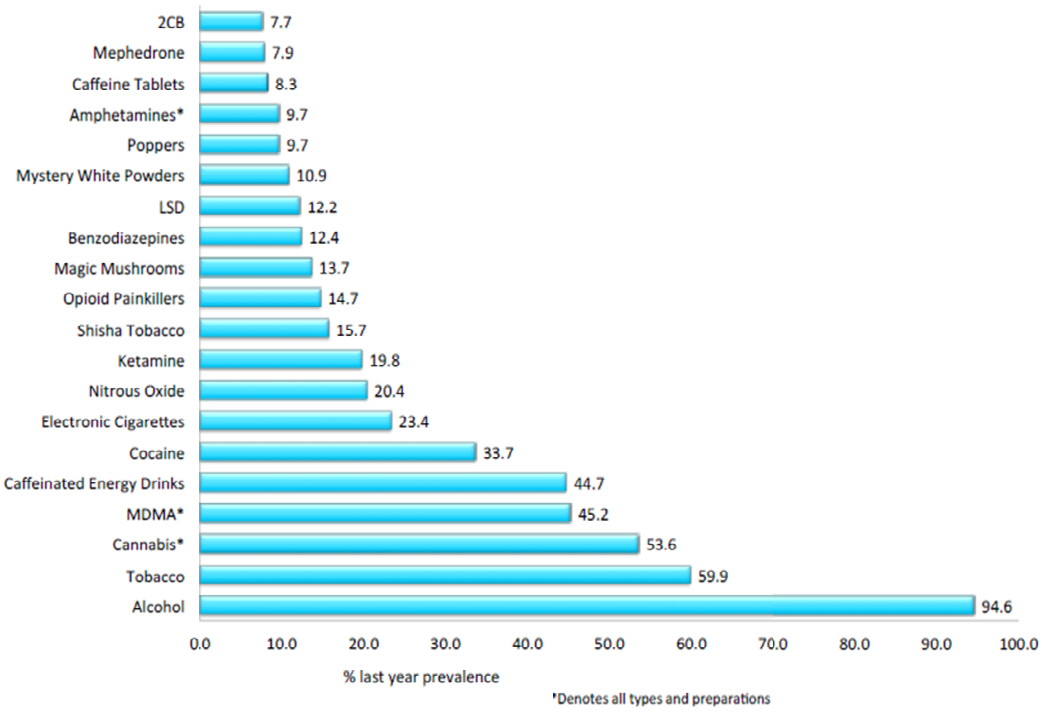
This study found no evidence for rapid sensitization or tolerance to mephedrone during a single binge dosing session. This is in contrast to the response to MDMA administration where repeated dosing converts the initial hypothermia to hyperthermia following subsequent injections, as measured by radiotelemetry (Rodsiri et al. 2011). The limited depletion of hypothalamic dopamine makes it impossible to completely exclude a role of this monoamine in mephedrone-induced hypothermia. However, since this response is unaffected by dopamine D_2 receptor blockade and prolonged by D_1 receptor antagonism (Shortall et al. 2013) the available evidence suggests that mephedrone-induced increases in dopamine efflux are unlikely to contribute to the drug-induced hypothermia, while modulation of central serotonergic neurotransmission plays a role in mediating both the hyperlocomotor and hypothermic effects of mephedrone. Although caution is required in attempting to translate the relevance of these findings in the rat to those in man they demonstrate the need to evaluate

the pharmacology and psychoactive effects of any new amphetamine analogues on a case-by-case basis and not rely on predictions from structural analogy.

Chapter 6 General Discussion

The synthetic cathinones received a lot of public attention in recent years, mostly due to media reports on 'legal high' related deaths. Mephedrone was the most popular of the cathinones to be used recreationally before the ban. Its popularity was thought to be due to its perceived likeness to MDMA, as well as the decline in purity of ecstasy tablets. Before the ban, mephedrone was found as a constituent of ecstasy tablets and remained as an active component of 'legal high' products after it was made a controlled substance. As part of a recent worldwide survey (2013) it has been confirmed that mephedrone is still amongst the top 20 recreational drugs used in the UK, but that it is not as popular as MDMA/ecstasy (Fig 6.1). Preclinical data on the behavioural, physiological and neurochemical effects of mephedrone have only been published since 2011. In light of this, the aim of this thesis was to examine mephedrone-induced changes in behaviour, body temperature or neurochemistry in the rat and to compare these changes to those observed following MDMA administration.

Recreational users liken mephedrone's psychostimulant effects to those of MDMA, and the chair of the UK's Advisory Council on the Misuse of Drugs is quoted as saying "[cathinone derivatives] are amphetamines by another name" (Dyer 2010). The main findings of this thesis however suggest that although mephedrone and MDMA share some similarities in their effects on behaviour and body temperature, they are also some key differences, suggesting some responses are mediated via similar but modified mechanisms.



6.1 Top 20 recreational drugs in the UK (Jan-Dec 2013)

The Global Drugs Survey was conducted during November and December in 2013 (n=7326 current drug users). Figure taken from (Winstock 2014).

6.1 Summary of findings

This body of work found that the neurochemical, behavioural and physiological effects of mephedrone in the rat include hyperactivity, hypothermia, cognitive deficits, anxiety-related behaviour and increased striatal dopamine efflux. Importantly, these effects appear to be occurring by mechanisms that are different from MDMA, cathinone and methcathinone. With regards to temperature, both cathinone and methcathinone caused a hyperthermic response, without any corresponding alteration in tail temperature, while MDMA and mephedrone induced hypothermia with a simultaneous decrease in tail temperature (Chapter 2). Additionally, the data presented in this same chapter and Chapter 5, demonstrate a role for dopaminergic, serotonergic and adrenergic systems in mephedrone-induced hypothermia which is attenuated by α_1-adrenoceptor (prazosin) and 5-HT_{1A} (WAY-100635) antagonism and prolonged by D_1 dopamine receptor antagonism (SCH 23390). Mephedrone also had no effect on body temperature when rats

were group-housed at the time of measurement, but pre-exposure to MDMA, or concomitant caffeine administration, caused an increase in rectal temperature (Chapter 2 and 4). 'Binge-style' administration of mephedrone (3 x 10 mg kg^{-1} at 2 h intervals) had no accumulative effect on mephedrone-induced hypothermia. Additionally, mephedrone-induced hypothermia is also abolished by 5-HT depletion and partially attenuated by dopamine depletion (Chapter 5).

Single cathinone and MDMA injections cause prolonged hyperactivity in rats, while mephedrone injection causes transient hyperactivity, with evidence for sensitisation to both cathinones and mephedrone following chronic intermittent dosing (a total of six injection administered twice weekly on consecutive days for three weeks, Chapter 3). Pre-exposure of rats to MDMA had no effect on mephedrone-induced hyperactivity after one or five injections, while co-administration of caffeine and mephedrone prolonged the hyperactive profile of mephedrone following acute injection (Chapter 4). Interestingly, 'binge-style' administration of mephedrone did not affect mephedrone-induced hyperactivity and 5-HT depletion (by bilateral i.c.v. injection) caused a decrease in the magnitude of mephedrone-induced hyperactivity, while dopamine depletion enhanced it (Chapter 5).

Cathinone, MDMA and mephedrone disrupted novel object discrimination but each compound also reduced exploration during the familiarisation trial (Chapters 3 and 4), which may be due to decreased attention rather than a specific memory deficit. Further studies using differing tasks would be required to conform this, for example the 5-choice serial reaction time task would assess visuospatial attention. Mephedrone was the only compound to reduce freezing in the retention trial of the conditioned emotional response task due to attenuation of hippocampal-dependent contextual association (Chapter 3). Mephedrone also increased 'anxiety-related' behaviour on the elevated plus maze (Chapter 4) which was enhanced by MDMA pre-exposure compared to mephedrone alone, without affecting the mephedrone-induced decrease in the number of head dips over the sides of the open arms. Co-administration of caffeine increased the time spent on the open arms of the maze compared to mephedrone alone and

reversed the decreased number of head dips observed in the mephedrone alone treated rats. Cathinone, MDMA and mephedrone had no effect on prepulse inhibition to acoustic startle.

The cathinones and MDMA had varying effects on post mortem tissue levels of the monoamines and their metabolites. Mephedrone (10 mg kg^{-1}) decreased 5-HIAA in hippocampal tissue dissected at 1 h post-injection (following twice weekly injections on consecutive days for three weeks), an effect not observed in mephedrone treated MDMA pre-exposed rats (Chapter 4). MDMA also decreased hippocampal 5-HT and 5-HIAA in MDMA pre-exposed rats, 1 h after the final MDMA injection (13 injections in total), while a dose of 4 mg kg^{-1} mephedrone increased hippocampal DOPAC and 10 mg kg^{-1} mephedrone and MDMA decreased DOPAC in tissue taken seven days post-injection (Chapter 3).

In post mortem tissue dissected at 2 h post-injection (Chapter 2), cathinone (4 and 10 mg kg^{-1}) increased hypothalamic DOPAC while the higher dose increased striatal dopamine, hypothalamic 5-HT and striatal 5-HT and 5-HIAA. Methcathinone (10 mg kg^{-1}) increased dopamine and HVA levels in the frontal cortex, hippocampus and striatum, as well as 5-HIAA in the striatum. In contrast, MDMA (4, 10 mg kg^{-1}) reduced 5-HT in the hippocampus, and the highest dose decreased DOPAC and HVA in the striatum, as well as decreasing 5-HIAA in the hippocampus, hypothalamus and striatum. Mephedrone had no effect on dopamine, 5-HT or their metabolites in tissue taken 2 h post-injection but it did cause an increase in plasma noradrenaline (Chapter 2). Despite mephedrone having no effect on *ex vivo* tissue levels of 5-HT or dopamine, it did increase *in vivo* striatal dopamine efflux in awake rats, the magnitude and duration of which was unaffected by 'binge-style' administration (Chapter 5).

6.2 Translational relevance

When conducting preclinical studies it is important to select a dose, and dose schedule, which is relevant to human use. In order to do this,

pharmacokinetic and pharmacodynamic information from both humans and animals is essential. The dose levels and schedules used in this study were selected to mimic those used by recreational users, as well as doses used in previously published studies where possible. MDMA and mephedrone are typically consumed at the weekend (Moore et al. 2013), so in Chapters 3 and 4 rats were injected twice weekly on consecutive days a week in an attempt to mimic this dosing pattern. Mephedrone users also typically re-dose in a session to maintain the desired effects of this short acting drug (Schifano et al. 2011) so a further study administered three injections of mephedrone over 4 h (Chapter 5).

A typical recreational dose of MDMA is one to two tablets (approximately 150 mg of MDMA), which, using allometric scaling, is the equivalent of approximately 5 mg kg^{-1} in the rat (Green et al. 2012). However, this does not take metabolism, bioavailability or protein binding into account. MDMA not only has a much greater plasma half-life in humans than in rats, it is more rapidly metabolised to form neurotoxic metabolites in rats than in humans, and increasing the dose from 1 to 2 mg kg^{-1} in humans will increase the plasma concentration of the drug four-fold (Baumann et al. 2009). Therefore, in Chapters 2 and 3, two doses of MDMA (4 or 10 mg kg^{-1}) were chosen. The lower dose was chosen to mimic that ingested by human users, and the higher dose was chosen to account for the greater rate of metabolism of MDMA in rats than humans. Chapter 4 again used a low dose (5 mg kg^{-1}) as this was found to produce sensitisation to MDMA in previous studies (Aberg et al. 2007) and is similar to doses used in man.

The most common route of recreational mephedrone administration is orally or by insufflation, with a typical dose being approximately 100-200 mg, but due to its short duration of action users will re-dose in an attempt to maintain its desirable effects and may take up 4 g in a single session (Schifano et al. 2011; Winstock et al. 2011). Since pharmacokinetic studies of mephedrone in humans have not yet been performed, it is difficult to infer a suitable dose for preclinical studies. However, post mortem examination of mephedrone-related fatalities shows that plasma mephedrone concentration in persons suffering a fatal overdose was in the region of 2000 ng ml^{-1}. This being similar to a fatal dose of MDMA and

given the modest differences in molecular weights of MDMA and the selected cathinones, it was logical to compare the same doses of all compounds in Chapter 2, and then to use the data from these studies to select appropriate doses for subsequent studies. Since we have little information on the metabolism and protein binding of mephedrone in humans, the interpretation of the preclinical data collected in this thesis with relation to recreational use is obviously limited. More information in man is needed but this may be difficult as researchers are less likely to conduct studies with this compound since it is now a banned substance.

6.3 Is mephedrone an 'amphetamine by another name'?

The data presented in this thesis suggest that mephedrone shares some characteristics with MDMA, such that both compounds decrease rectal and tail temperature in individually housed rats, induce hyperactivity, disrupt learning in the familiarisation trial of the NOD task, increase anxiety-like behaviours in the elevated plus maze and increase striatal dopamine release. Additionally, co-administration of caffeine with mephedrone causes increased hyperactivity and converts mephedrone-induced hypothermia to hyperthermia, both of which are characteristic of concomitant MDMA and caffeine administration (Vanattou-Saifoudine et al. 2012). However, caution should be taken when comparing mephedrone to existing psychoactive substances. Taking a closer look at the data shows that the effects of mephedrone are not easily predicted from previous studies with MDMA, cathinone or methcathinone. For instance, mephedrone induces hypothermia and a decrease in peripheral tail temperature, while cathinone and methcathinone administration both cause increased rectal temperature with no change in tail temperature (Chapter 2), and mephedrone-induced hypothermia is transient and of a lower magnitude than the hypothermia observed following MDMA administration to individually housed rats.

Furthermore, based on the data presented in Chapter 2 it appears that mephedrone is eliciting temperature changes via different mechanisms to

MDMA. Mephedrone-induced hypothermia is enhanced by α_1-adrenoceptor and D_2 dopamine receptor antagonists, while MDMA-induced hypothermia is blocked by D_2 receptor antagonism (remoxipride) and potentiated and prolonged by the α_2-adrenoceptor antagonist, BRL 44408 (Green et al. 2005). As previously stated, hyperthermia is potentially fatal adverse effect of MDMA. Interestingly, both group housing and 'binge-style' repeated administration of MDMA cause hyperthermia in rats, but neither of these conditions affected temperature in mephedrone treated rats.

As previously stated, repeated MDMA administration to rats produces behavioural sensitisation to other 'amphetamine-like' psychostimulants, which is thought to result from enhanced dopamine release in the nucleus accumbens and striatum (Robinson and Becker 1982; Bradbury et al. 2012) and this is both dose level and administration interval dependent (Kalivas et al. 1993). Therefore, further evidence for the dissimilarity between mephedrone and MDMA is the lack of cross-sensitisation to the hyperlocomotor and 'anxiogenic-like' effects of a challenge dose of mephedrone in rats previously administered MDMA (Chapter 4).

Mephedrone and MDMA cause acute release of dopamine and 5-HT measured by *in vivo* microdialysis (Kehr et al. 2011; Baumann et al. 2012; Bradbury et al. 2013). It is well established that a high-dose or repeated administration of MDMA causes long-term 5-HT neurotoxicity in rats (see section 1.3.3). Acutely MDMA induces 5-HT release resulting in tissue 5-HT levels being reduced after 2 h but returning to baseline levels within 24 h. In the following seven days, 5-HT levels begin to decline again resulting in 5-HT depletion (Schmidt et al. 1987; Connor et al. 1998). In this thesis MDMA did not cause such neurotoxicity but this may be due to the dosing regime, the time at which tissue was taken or strain of rat used. However, acutely (2 h post-injection) MDMA (10 mg kg^{-1}) decreased striatal tissue levels of dopamine and 5-HT metabolites while also decreasing 5-HIAA in the hypothalamus and hippocampus. In the same timescale, mephedrone (4 and 10 mg kg^{-1}) decreased hippocampal tissue 5-HT levels only. However, in complete contrast, cathinone increased striatal dopamine, 5-HT AND 5-HIAA as well as hypothalamic 5-HT, while methcathinone

increased dopamine and HVA in the frontal cortex, hippocampus and striatum, and also increased 5-HIAA in the striatum.

These contrasts are unsurprising due to the differences in receptor binding profiles between mephedrone and MDMA, cathinone and methcathinone. Mephedrone, like MDMA, is a non-selective monoamine re-uptake inhibitor but cathinone and methcathinone are catecholamine reuptake inhibitors and releasers (Carvalho et al. 2012). Mephedrone binds to the 5-HT_{2A} receptor and α_{1A}- and α_{2A}-adrenoceptors with low micromolar affinity, but has very little affinity for 5-HT_{1A}, 5-HT_{2C} or dopamine receptors (Hadlock et al. 2011; Baumann et al. 2012; Lopez-Arnau et al. 2012; Martinez-Clemente et al. 2012; Eshleman et al. 2013; Simmler et al. 2013). MDMA binds to the α_2-adrenoceptor with a similar affinity as mephedrone, and has little affinity for 5-HT_{2C} or dopamine receptors, but also binds to α_{1-} and β-adrenergic, 5-HT_1 and M_2 muscarinic receptors (Battaglia et al. 1988).

6.4 Future studies

All new psychoactive drugs are being taken by users without any important information about their short and long term effects or interactions with other pharmacological agents. It is important that each novel substance is treated as such and investigated on a case-by-case basis, however, it is impossible to keep up with the rate at which these new drugs are emerging, as well as with the rapidly changing patterns of recreational drug use and co-administration. When the first of the studies presented in this thesis was conducted, at the height of mephedrone's popularity, there were no preclinical publications on the pharmacological effects of mephedrone. While the data presented in this thesis provide interesting insight into the effects of mephedrone on certain aspects of behaviour and neurochemistry in animal models, there are a number of studies that would be beneficial as a continuation of the work presented in this thesis.

The most common and consistent effects of mephedrone as described in this thesis, and in various other studies, are hyperactivity and hypothermia

(Kehr et al. 2011; Lisek et al. 2012; Lopez-Arnau et al. 2012; Marusich et al. 2012; Motbey et al. 2012; Wright et al. 2012; Gregg et al. 2013; Martinez-Clemente et al. 2013; Miller et al. 2013). The data presented in this thesis have indicated a role for the α_1-adrenoceptor, 5-HT_{1A}, dopamine D_1 receptors in the mephedrone-induced hypothermic response, and a study by another group has shown that D_1 dopamine receptor antagonist pre-treatment inhibits mephedrone-induced hyperactivity but D_2 dopamine receptor antagonist pre-treatment enhances this response (Lisek et al. 2012). Given that mephedrone appears to induce elements of the 5-HT syndrome (Baumann et al. 2012), and that 5-HT_2 receptor antagonism as well as systemic p-chlorophenylalanine administration to the mouse (Lopez-Arnau et al. 2012) or central 5,7-DHT administration to rats inhibit mephedrone-induced hyperactivity (Chapter 5), it would be interesting to investigate the effects of selective 5-HT receptor antagonists on this acute hyperlocomotor response in the rat.

Following from this, there are a number of studies investigating the effects of mephedrone on 5-HT and/or dopamine release in the nucleus accumbens (Kehr et al. 2011; Baumann et al. 2012; Wright et al. 2012) and striatum (Chapter 5). This may account for any acute re-inforcing effects of mephedrone but as previously stated this does not account for any temperature effects of mephedrone. The hypothalamus plays an important role in homeostatic functions, including regulation of body temperature. While this thesis has demonstrated the role of various receptors in the hypothermic response to mephedrone, whether this is occurring through peripheral or central nervous system actions has not yet been distinguished. In order to further differentiate the peripheral actions of mephedrone from its central actions on altering temperature it would be beneficial to study the effects of mephedrone on *in vivo* neurotransmitter release within the hypothalamus using microdialysis techniques, preferably following rapid repeated dosing as described in Chapter 5.

Finally, since mephedrone users tend to take other substances concurrently with mephedrone (Carhart-Harris et al. 2011; Corkery et al. 2012), and that the data presented in this thesis indicate an increased effect of previous MDMA or concomitant caffeine use on mephedrone-

induced responses, it would be beneficial to further investigate the effects of other recreational drugs on mephedrone use. For example, as mephedrone is commonly taken at night clubs, there is an increased likelihood that it will be taken with alcohol. Additionally, the majority of mephedrone users have a lifetime prevalence of cocaine use (for example 92 % of 947 mephedrone users admit to previous use of cocaine; Winstock et al., 2011a) and although mephedrone has become an illegal substance it is still found in products sold as 'legal highs' along with other cathinones such as 3,4-methylenedioxypyrovalerone (MDPV) and MDPV has been identified post-mortem in a number of mephedrone-related deaths (Corkery et al. 2012). Based on this information it would be interesting to examine the behavioural and long-term neurochemical effects of previous cocaine use on subsequent mephedrone administration in rats, as well as ethanol or MDPV administration concomitantly with mephedrone, using a similar protocol as described in Chapter 4, to try to further explain the adverse effects of mephedrone not observed when it is administered alone.

6.5 Conclusion

Although mephedrone users compare its effects to MDMA, the data presented in this thesis suggests that these two compounds, as well as cathinone and methcathinone, have a different, but related pharmacology. Like MDMA, mephedrone acts via the monoamine systems to elicit a wide range of behavioural effects including hyperactivity, and hypothermia. However, in stark contrast to MDMA, mephedrone does not appear to cause neurotoxicity in the rat, at least under the conditions used herein. Further studies investigating the effects of mephedrone in human users, including its pharmacokinetics, are essential in fully understanding the pharmacology of this drug.

Equally, while MDMA and mephedrone are still amongst the top 20 most popular drugs in the UK (Winstock 2014), the recreational drug scene is constantly changing, with novel substances constantly emerging at a rate much too quick for the appropriate research to be conducted before human use. Baring this in mind, the legal bans on substances based on generic

chemical structures that have been put in place further impedes any scientific research that needs to be conducted as research is limited to laboratories that have licences to work with these substances. Although the intention of these generic bans is to protect against human use, historically this does not prevent people from taking them, and with the added limitations placed on researching these chemicals, there is little information available on any potential harms that they may cause. Additionally, purchasing these substances illegally increases the risk of taking impure mixtures of several different drugs, which may result in severe adverse events or death. Instead of generically banning these substances it would be more beneficial to educate people based on scientific evidence on the risks associated with taking unknown or new drugs in a way that they will understand and be receptive to, in order to limit any potential harms of these novel substances.

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