ELUCIDATING THE NEUROPHARMACOLOGICAL PROPERTIES OF THE NOVEL PSYCHOACTIVE SUBSTANCE AND SYNTHETIC CATHINONE, MEPHEDRONE

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Abstract

Mephedrone (4-methylmethcathinone) is an illicit psychoactive stimulant and synthetic cathinone which gained prominence in the UK as a “legal high” circa 2008, subsequently being made illegal following media reports of adverse effects and links to several fatalities, as well as its structural similarity to amphetamine. Today, mephedrone remains in recreational use worldwide, often consumed alongside traditional illicit substances such as methamphetamine and gamma-hydroxybutyrate (GHB), or legal drugs such as caffeine and alcohol. In rats, co-administration of caffeine with MDMA (3,4-methylenedioxymephampethamine), has been shown to potentiate the elevation of extracellular 5-HT brain levels, a neurochemical correlate of the serotonin syndrome. In humans, this syndrome is characterised by adverse physiological effects including fever, agitation and hypertension.

Despite increased elucidation of its pharmacological profile since 2008, there remains a paucity of data on mephedrone’s behavioural and neurochemical effects, particularly when combined with caffeine. The present thesis sought to somewhat mitigate this deficit. First, a repeated dosing regimen was designed to assess the acute effects of repeated mephedrone administration, with and without caffeine, on behavioural and physiological measures in adolescent rats, and any lasting changes in anxiety, cognition and microglial activation in adulthood. Second, following the observation of hyperthermia and stereotyped behaviours in adolescent rats, an *in vivo* microdialysis study was designed to elucidate whether this apparent serotonin syndrome was elicited via increased downstream activation of postsynaptic 5-HT_{1A} receptors by endogenous 5-HT. In sum, mephedrone elicited changes in body temperature and locomotor hyperactivity in both studies (with tolerance to the latter developing throughout the one-week binge-type dosing period in study 1). In each case, caffeine converted mephedrone-induced hypothermia to hyperthermia, and enhanced mephedrone-induced stereotyped behaviours. Pre-administration of the 5-HT_{1A} receptor antagonist WAY-100,635 failed to prevent any of these effects, and in fact sped the onset of the hyperthermic response, perhaps via downstream effects

following binding to 5-HT_{1A} autoreceptors in the dorsal raphe nuclei. Nonetheless, no lasting effects of mephedrone, caffeine, or the combination of each, were observed on recognition memory, anxiety, sensorimotor gating, conditioned freezing or hippocampal microglial activation.

Publications

Abstracts

O'Hara L, Akingbade O, Fisher A, Marriott H, Morris PO, Perera MGA, Green AR, Fone KCF, King MV. (2019). Co-administration of mephedrone and caffeine enhances stereotyped behaviour in adolescent rats, without long-term changes in cognition or hippocampal microglial activation. *Journal of Psychopharmacology,* **33**, A86-87.

O'Hara L, Adam L, Watson DJG, Spicer CH, Bonsu N, Lankester O, Martinez W, Sawicka G, Sritharan A, Chilinski K, Gomes L, Ratnasingham M, Savage B, Green AR*, Fone KCF, King MV (2021). *ARG died September 2020. Additive effects of mephedrone and caffeine on locomotor activity, temperature, stereotyped behaviour and striatal c-Fos expression in rats are unaffected by 5-HT_{1A} receptor blockade. ***In press.***

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Thanks also to SARS-CoV-2, if not for your contribution, I would have written this page twelve months ago.

Abbreviations

2C-B – 4-Bromo-2,5-dimethoxypenethylamine

2-MMC – 2-methylmethcathinone

3-MMC – 3-methylmethcathinone

5-HIAA – 5-hydroxyindoleacetic acid

5-HT – 5-hydroxytryptamine

5,6-DHT – 5,6-dihydroxytryptamine

5,7-DHT – 5,7-dihydroxytryptamine

6-OHDA – 6-hydroxydopamine

ACMD – Advisory Council on the Misuse of Drugs

ANOVA – Analysis of variance

ATP – Adenosine triphosphate

BBB – Blood-brain barrier

BDNF – Brain-derived neurotrophic factor

CFR – Conditioned Freezing Response

CPP – Conditioned place preference

CSPP – Cortico-striato-pallidopontine

CX – Cortex

CYP2D6 – Cytochrome P450 2D6

DA – Dopamine

DAT – Dopamine transporter

DEA – Drug Enforcement Agency

DOI – 2,5-Dimethoxy-4-iodoamphetamine

DOPAC – Dihydroxyphenylacetic acid

DR – Dorsal raphe

ED_{50} – Median effective dose

EPM – Elevated plus maze

ESCAPE – European Syringe Collection and Analysis Project Enterprise

EU – European Union

FCX – Frontal cortex

Fig. – Figure

FR – Fixed ratio

GABA – Gamma-aminobutyric acid

GBL – Gamma-butyrolactone

GHB – Gamma-hydroxybutyrate

g kg^{-1} – Grams per kilogram of body weight

HIP – Hippocampus

HIV – Human immunodeficiency virus

HPLC-ED – High performance liquid chromatography with electrochemical detection

HVA – Homovanillic acid

HYP – Hypothalamus

ICSS – Intracranial self-stimulation

IC_{50} – Half maximal inhibitory concentration

i.c.v. – Intracerebral ventricular

i.m. – Intramuscular

i.p. – Intraperitoneal

ITI – Inter-trial interval

i.v. – Intravenous

K_B – Equilibrium dissociation constant

K_i – Inhibitory constant

LC – Liquid chromatography

LD_{50} – Median lethal dose

LDH – Lactate dehydrogenase

LGBT – Lesbian, gay, bisexual and transgender

LMA – Locomotor activity

LSD – Lysergic acid diethylamide

MAO – Monoamine oxidase

MDA – 3,4-methylenedioxyamphetamine

MDMA – 3,4-methylenedioxymethamphetamine

MDPV – Methylenedioxypyrovalerone

Meph – Mephedrone

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

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

mPFC – Medial prefrontal cortex

MS – Mass spectrometry

MSM – Men who have sex with men

MWM – Morris water maze

NAcc – Nucleus accumbens

NET – Noradrenaline transporter

NMDA – N-methyl-D-aspartate

NOD – Novel object discrimination

NOL – Novel object location

NPS – Novel psychoactive substance

OFT – Open field test

ONS – Office of National Statistics

PCA – Perchloric acid

PCP – Phencyclidine

PDE5 – Phosphodiesterase 5

pK_B – Negative logarithm of K_B value

PMA – Para-Methoxyamphetamine

PMMA – Para-Methoxymethamphetamine

PPI – Prepulse inhibition of the acoustic startle response

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

s.c. – Subcutaneous

SEM – Standard error of the mean

SERT – 5-HT transporter

SN – Substantia nigra

SSRI – Selective serotonin reuptake inhibitor

STR – Striatum

TH – Tyrosine hydroxylase

TPH – Tryptophan hydroxylase

UN – United Nations

USV – Ultrasonic vocalisation

VMAT – Vesicular monoamine transporter

VTA – Ventral tegmental area

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Chapter 1. General Introduction

1.1 Novel Psychoactive Substances

1.1.1 Commercial and legislative history

1.1.1.1 The decline in availability of MDMA

In 2009, there was a market shortage of the illicit recreational stimulant drug 3,4-methylenedioxymethamphetamine (MDMA) **(Fig. 1.1)**. This was largely attributable to the decreased availability of the precursor safrole, a naturally occurring constituent of saffrasas oil, a vegetable product sourced from tropical forests. In 1999, regulators within the United Nations (UN) and Europe placed a ban on the unlicensed production and distribution of safrole, in an effort to curb MDMA synthesis and thereby recreational use.

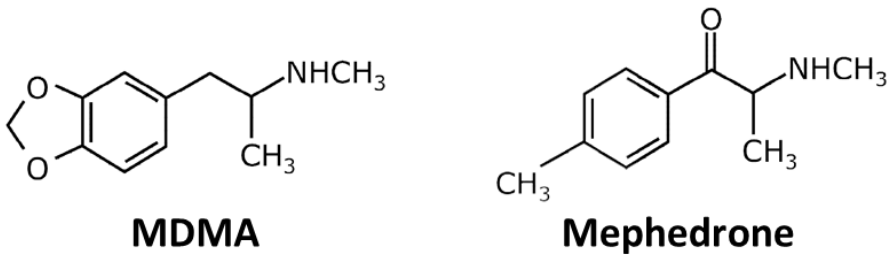


Figure 1.1 Chemical structures of MDMA and mephedrone.

However, the 2009 shortage likely resulted more directly from an event in Thailand the previous year, when 50 tons of saffrasas oil were seized, translating to around half of the annual global manufacture of "Ecstasy" (Nutt, 2020). An immediate effect was observed in the Dutch Ecstasy market in July 2008, from which point the MDMA content rapidly decreased across successive months (Brunt *et al.*, 2011) **(Fig. 1.2)**.

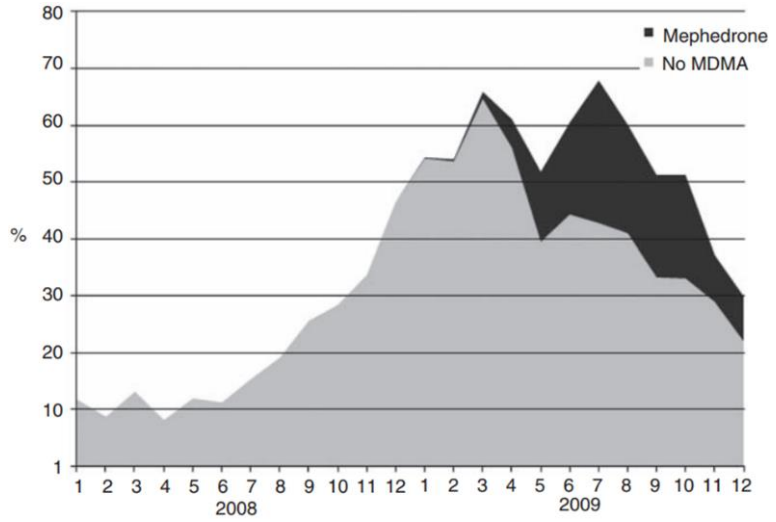


Figure 1.2 Police seizures in The Netherlands, 2008 and 2009. Seizures in The Netherlands indicated a decline in MDMA content in Ecstasy products, and the increase in use of mephedrone as a substitute or adulterant, across 2008 and 2009, given as a percentage of tablets per month (taken from Brunt et al., 2011).

Reduction in safrole's availability presented MDMA manufacturers with two solutions: finding an alternative means of synthesis; or changing the composition of Ecstasy products. Attempts at alternative synthesis led to the employment of anethole, a compound chemically similar to safrole, found in toiletries and cosmetic products. However, synthesis with anethole leads to the production of paramethoxyamphetamine (PMA) or paramethoxymethamphetamine (PMMA), which are absorbed more slowly than MDMA, and may therefore precipitate re-dosing in recreational users and increase the likelihood of adverse effects which include hyperthermia, seizure and death. The replacement of MDMA with PMA/PMMA in Ecstasy tablets precipitated a rapid increase in Ecstasy-related deaths in England and Wales, peaking at 29 in 2013, relative to zero in 2010, according to the UK Statistics Authority's executive office (ONS, 2019). In Israel, between February 2007 and January 2008, of 32 fatalities which tested positive for amphetamines, 24 involved PMMA and PMA. Of these 24 cases MDMA and MDA (3,4-methylenedioxyampethamine) were detected as co-present drugs in 45.8 and 37.5% of sample, respectively (Lurie *et al.*, 2012).

In parallel, many Ecstasy products, historically comprising MDMA as their chief psychoactive constituent, underwent a notable increase in adulteration. In 2009, a seizure in The Netherlands reported less than 50% of Ecstasy tablets comprised MDMA as their main constituent, relative to the 90% purity of previous years (Brunt *et al.*, 2011). The most common adulterant of these products was mephedrone, a cheap alternative stimulant (EMCDDA, 2010a) with effects akin to those of cocaine and/or MDMA (Winstock *et al.*, 2011a). Mephedrone is a synthetic cathinone and novel psychoactive substance (NPS) which around this time appeared to become the most popular of the synthetic cathinones in its own right (Winstock *et al.*, 2011b).

1.1.1.2 Mephedrone's establishment as a recreational drug

In 2009, an online survey of 2,289 experienced polydrug users revealed 42% used mephedrone on at least one occasion, whilst around 30% used it at least as frequently as every two weeks (Winstock *et al.*, 2011b). Between 2010 and 2012, mephedrone was also identified as the most popular NPS in post-mortem and criminal casework, present in 106 of 203 cases, with 4-methylethcathinone at a distant second (28 cases) (Elliott & Evans, 2014). At the height of its popularity, mephedrone use was sensationalised in the UK press and media. Numerous fatalities and severe casualties linked to ingestion of mephedrone were later revealed to be consequential of either other substances or natural causes (Measham *et al.*, 2010; Sare, 2011), whilst a report in a popular tabloid newspaper of a mephedrone user ripping off his scrotum was subsequently determined an internet hoax (Davey *et al.*, 2010).

1.1.1.3 Legal status

Novel psychoactive substances (NPS) are defined as those not scheduled for prohibition in either United Nations Single Conventions (1961, 1971) on Narcotic Drugs (UNODC, 1972). Prior to 2010, much reference was made to NPS as "legal highs" (Laurance, 2010), denoting them as substances synthesised with the intent of

replicating or surpassing the psychoactive effects of drugs already legislatively prohibited. Mephedrone was reportedly even referred to by the chair of the UK-based Advisory Committee on the Misuse of Drugs (ACMD) as “an amphetamine by another name” (Dyer, 2010). Circumvention of legislation in this manner is not a recent phenomenon. Prior to its 2001 scheduling, for instance, 2,5-dimethoxy-4-bromophenetylamine (2C-B) was promoted as a legal alternative to the chemically-related MDMA (Dean *et al.*, 2013). It is to some degree in respect of the transient legality of these substances (Karila & Reynaud, 2011), and perhaps their misleading advertisement (in the case of mephedrone, as “plant food”; see section **1.1.4.1**) (Gibbons & Zloh, 2010) that consumers might consider them safer than more traditional or conventional illicit drugs.

Between April 2010 and July 2012 all ‘legal highs’ became illegal in the UK (ACMD, 2010), US (Haggin, 2012), and across Europe (EMCDDA, 2011). In the UK, this entailed Class B scheduling on 16 April 2010 by Prime Minister David Cameron (HMSO, 2010). Disappointingly, it may be considered that the UK ban on mephedrone was informed more by sensationalised and inaccurate media portrayals than by scientific literature. Nonetheless, there is evidence mephedrone remained available for illicit use, from the immediate post-ban period to the present day (Brandt *et al.*, 2010b; McElrath & O'Neill, 2011; Ayres & Bond, 2012; Van Hout & Bingham, 2012; Kelly *et al.*, 2013; Yamamoto *et al.*, 2013). A timeline illustrating the history of mephedrone use is provided in **Fig. 1.3**.

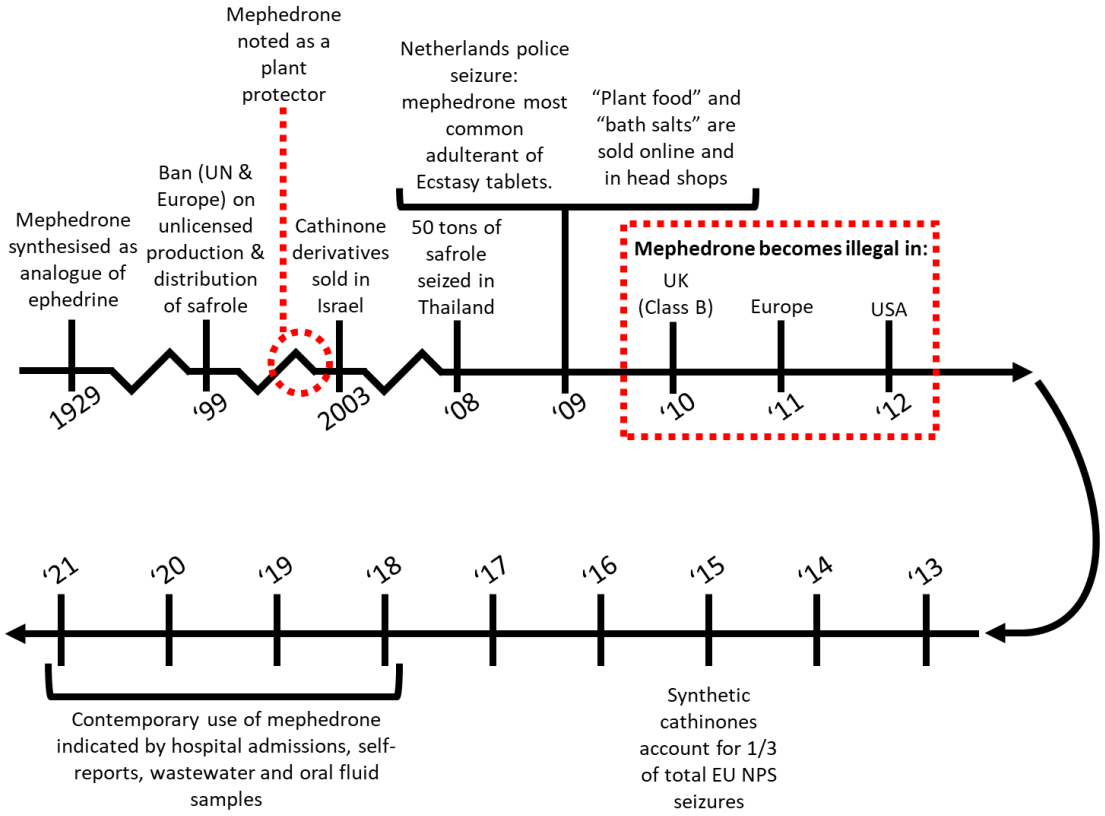


Figure 1.3 Timeline depicting the emergence of mephedrone as a recreationally used drug, spanning from synthesis (1929) to present day (2021).

1.1.2 Cathinone and synthetic cathinones

NPS are sometimes categorised in relation to their action to produce the psychotropic effects of another classified substance. In the cases of the synthetic cannabinoids and cathinones, these aim to emulate the effects of *Cannabis* and cathinone (or psychostimulants more generally), respectively.

Synthetic cannabinoids, sold under such street names as "Spice" and "Black Mamba", are specifically produced to induce effects similar to *Cannabis*, and have been used as such since at least 2004 (Debruyne & Le Boisselier, 2015). Similarly, synthetic cathinones are designed to produce effects comparable to cathinone, a cardinal psychoactive constituent of *Catha edulis* (or "Khat" plant) leaves (Valente *et al.*, 2014).

Consumption of this evergreen shrub, originally native to the Horn of Africa (Gebissa, 2010; Valente *et al.*, 2014) and thereafter (Alles *et al.*, 1961) the Southern Arabian Peninsula (Sheikh *et al.*, 2014), has in recent years been evidenced in diaspora communities resident in European countries (Bongard *et al.*, 2015). For several centuries khat has been utilised, via chewing or consumption as tea (Feyissa & Kelly, 2008) as a 'social lubricant' (Valente *et al.*, 2014; El-Menyar *et al.*, 2015) and aid to religious observance (Alles *et al.*, 1961). More tentatively, it is fabled as a medicinal choice of withstanding fatigue in warriors (Khatib *et al.*, 2013), including those of Alexander the Great (Zahran *et al.*, 2014).

1.1.2.1 Mephedrone as a synthetic cathinone

Mephedrone (4-methylmethcathinone), first synthesised in 1929 by Saem de Burnaga Sanchez as a homologue of ephedrine (Green *et al.*, 2014; Nutt, 2020), is a synthetic cathinone and close structural analogue of methamphetamine (den Hollander *et al.*, 2014b). It is suggested that in the early 2000s, Israeli scientists working at an insecticide company successfully used mephedrone to disrupt the brain activity of aphids, allowing for the protection of plants and the later marketing of mephedrone as "plant food" (Nutt, 2020). The legal sale of synthetic cathinones for recreational use appears to have begun in Israel around this time (Green *et al.*, 2014; Nutt, 2020), with capsules of "Hagitat" (translated from Hebrew as "Khat party") reportedly being sold in convenience stores in late 2003, advertised as natural stimulants and aphrodisiacs (Bentur *et al.*, 2008).

In the UK, the sale of mephedrone as "plant food" or "bath salts" which were "not fit for human consumption" allowed for a bypass of regulation under the Medicines Act 1968. These products were variably composed of mephedrone, MDPV and methylone, as well as a number of other compounds (Glennon, 2014).

1.1.2.2 Synthetic cathinones as established recreational drugs

In 2015, synthetic cathinones accounted for 33% of total NPS seizures in the European Union (EU) (EMCDDA, 2017), a preference demonstrated in 2016 and 2017, when they accounted for the largest group of newly-synthesised substances **(Fig. 1.4)**. Despite in 2015 failing to rank among the top five most commonly seized of the synthetic cathinones, recreational preference for mephedrone and mephedrone-like substances was indicated in that two of these top five comprised structurally-related derivatives (2-MMC and 3-MMC); products which may also have been sold as "mephedrone" (EMCDDA, 2017). Further support derives from LC-MS/MS analysis of hair samples in Paris between 2012 and 2017, citing mephedrone as the joint-most frequently detected of all synthetic cathinones in patients presenting to hospital (Larabi *et al.*, 2019). Similarly, another structural derivative, mexedrone, presented in urine of 11 unwitting NPS-consuming patients declaring polysubstance use (Roberts *et al.*, 2017), indicating use of this compound as an adulterant during the manufacturing phase. A lack of regulation renders the composition of mixtures sold as bath salts or plant food unpredictable, and concentrations of the main ingredients (mephedrone, MDPV and methylone) fluctuate, with potentially adverse consequences. Each ingredient possesses a similar though unique pharmacological profile which elicits similar changes in neurochemical, psychological, and physiological measures. As such, their co-use may elicit a synergistic or additive effect on these measures (Tallarida, 2011), as has been demonstrated in mice (Allen *et al.*, 2019).

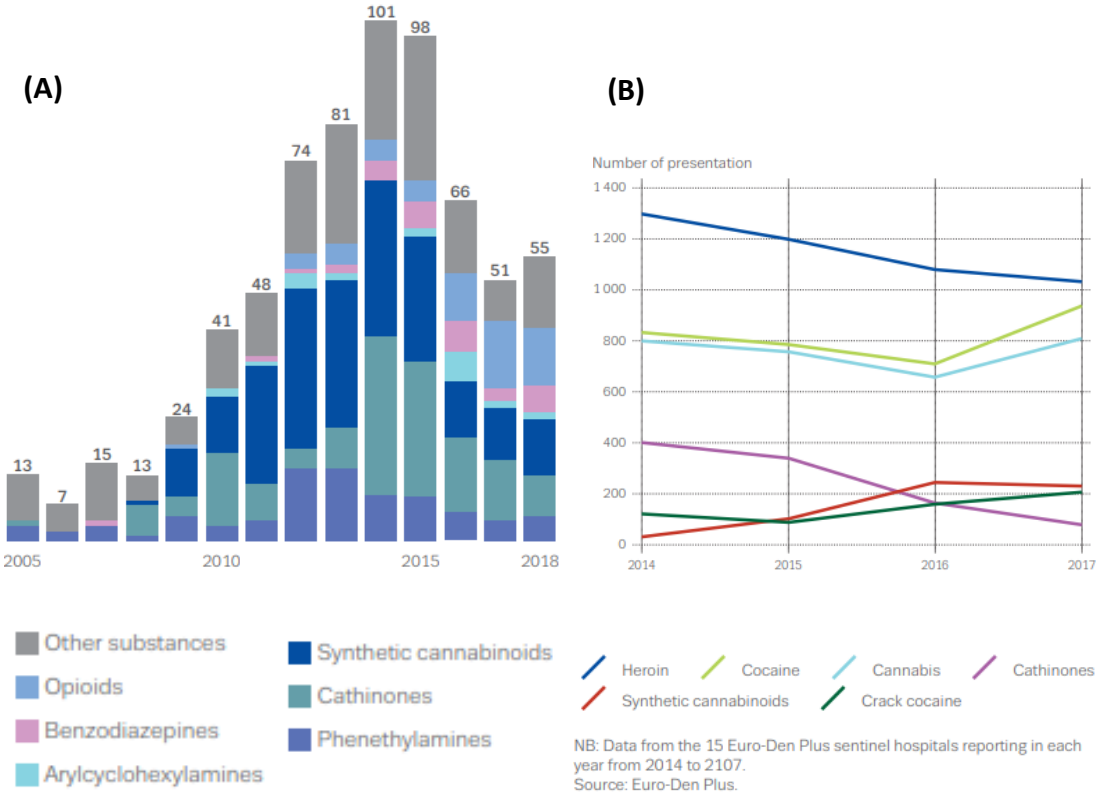


Figure 1.4 Reports to EU Early Warning System of unique NPSs between 2005 and 2018. Between 2015 and 2018, despite a decrease in newly-synthesised synthetic cathinones, their production was evidenced to have continued. Reports to EU Early Warning System of unique NPSs increased rapidly between 2010 and 2015, with most newly-marketed substances identified as synthetic cathinones, cannabinoids or phenethylamines (A); a decrease in trends in the number of presentations to sentinel hospitals related to selected drugs (B). Hospital presentations relating to synthetic cathinone use decreased between 2014 and 2017, from ~ 400 to < 100. Figures taken from EMCDDA (2019).

1.1.2.3 Chemical structure

Cathinone and its synthetic derivatives exhibit structures similar to those of amphetamines, differing through the attachment of a ketone to the beta position of the amino alkyl chain on the phenyl ring (Prosser & Nelson, 2012), and are therefore referred to as β-keto amphetamines (Zaitsu *et al.*, 2011). Cathinone, methcathinone

and methylone, for example, are the β-keto analogues of amphetamine, methamphetamine and MDMA, respectively **(Fig. 1.5)**.

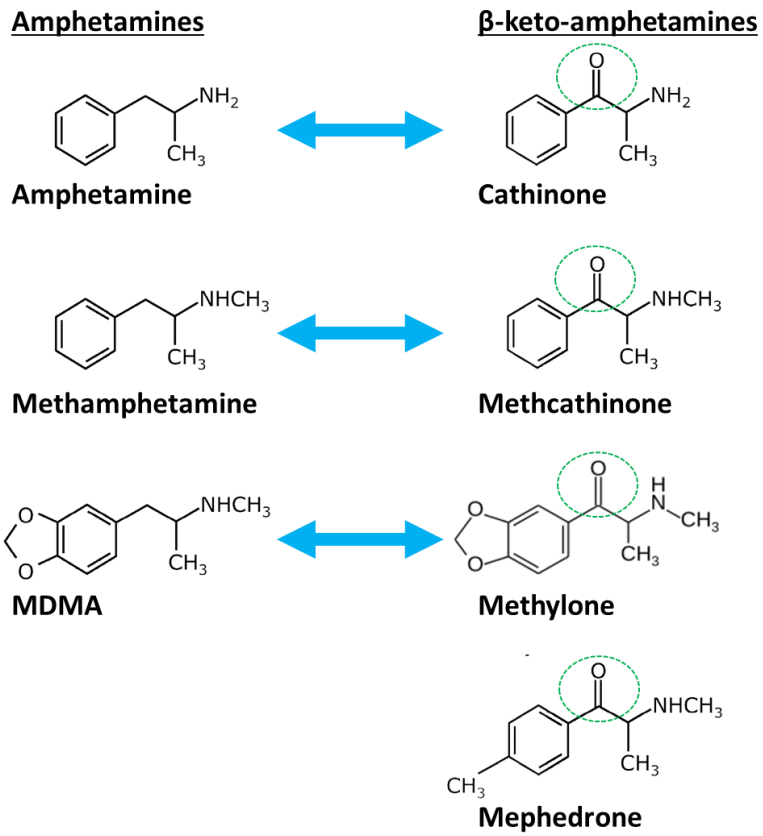


Figure 1.5 Chemical structures of some amphetamines and β-keto amphetamines. Cathinone, methcathinone and methylone are structural analogues (⟷) of amphetamine, methamphetamine and MDMA, respectively, differing through the addition of a ketone functional group (◌). At present, there is no commonly used, known structural amphetamine analogue of mephedrone.

The variety of synthetic cathinones marketed to date are produced by substitutions of cathinone's core structure, and can be categorised into four typical categories. These substitutions include: alkyl substitutions at the α-carbon of the side chain and/or in the benzyl ring, producing mephedrone; the addition of a methylenedioxy benzyl ring, producing methylone; the addition of an *N*-pyrollidinal moiety, producing alpha-pyrrolidinopentiophenone (α-PVP); or the addition of a methylenedioxy benzyl ring

and *N*-pyrollidinal moiety, producing MDPV (Valente *et al.*, 2014; Nobrega & Dinis-Oliveira, 2018; Calinski *et al.*, 2019) **(Fig. 1.6)**.

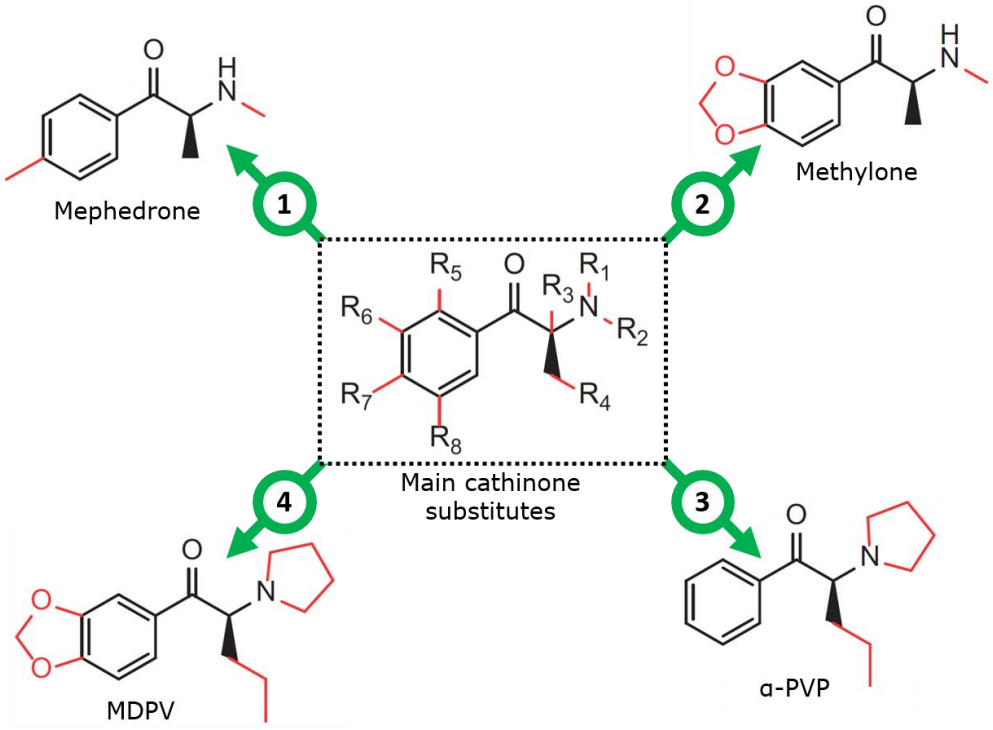


Figure 1.6 Synthetic cathinones can be derived from cathinone through a variety of modifications to cathinone's core structure. Cathinone derivatives can be synthesised via: alkyl substitutions at the α-carbon of the side chain and/or in benzyl ring (**1**); addition of a methylenedioxy benzyl ring (**2**); addition of an *N*-pyrollidinal moiety (**3**); or addition of a methylenedioxy benzyl ring and *N*-pyrollidinal moiety (**4**). Figure adapted from Nobrega & Dinis-Oliveira (2018).

1.1.3 Recreational use

The popularity and usage trends of illicit drugs and drug products are tangible from analyses of user self-reports, online surveys, amnesty samples, police seizures and wastewater-based epidemiology (Castiglioni *et al.*, 2014; Castrignanò *et al.*, 2017). Congruence of data obtained via these techniques informs refinement of investigative scope in favour of researching contemporarily relevant substances.

1.1.3.1 Measurements of recreational use

An early insight into the recreational use of mephedrone in the UK is provided by data of an anonymous online survey of patrons of dance music and clubbing venues. Of 2,289 participants, 947 (41.3%) reported having used mephedrone on at least one occasion; with 15.1% of these on at least a weekly basis (Winstock *et al.*, 2011b).

Since 2010, mephedrone has been detected in wastewater samples taken from urban cities of countries in Europe (Celma *et al.*, 2019), including Denmark (Bade *et al.*, 2017), Italy (Castiglioni *et al.*, 2015; Gonzalez-Marino *et al.*, 2016), Poland (Styszko *et al.*, 2016; Sulej-Suchomska *et al.*, 2020), Spain (Fontanals *et al.*, 2017) and the UK (Mwenesongole *et al.*, 2013; Castrignano *et al.*, 2016; Castrignano *et al.*, 2018), as well as in UK drinking water (Peng *et al.*, 2019) and urine samples (Archer *et al.*, 2014), river samples of Spain (Fontanals *et al.*, 2017), hair samples of France (Martin *et al.*, 2012) and police seizures in The Czech Republic (Jurásek *et al.*, 2020). Mephedrone has also been detected in South Africa (Archer *et al.*, 2018), Australia (Chen *et al.*, 2013; Tscharke *et al.*, 2016) and The Maldives (Fallati *et al.*, 2020), and has been sold in India since at least 2013 (Palkar & Kumthekar, 2015).

Developing techniques, including UHPLC-MS/MS for analysis of oral fluid (Malaca *et al.*, 2019) and nail samples (Busardò *et al.*, 2020), LC-MS/MS for hair samples (Freni *et al.*, 2019), electrochemical detection (Razavipanah *et al.*, 2018) and multiplexed quantitative detection for the analysis of urine samples (Muhamadali *et al.*, 2019), as well as X-ray powder diffraction for the analysis of seized drug samples (Jurásek *et al.*, 2020) are becoming increasingly useful tools in the detection of mephedrone, the latter having recently been used to successfully determine the high purity of a mephedrone sample seized by police in The Czech Republic (Jurásek *et al.*, 2020). Likewise, detection of mephedrone in its (*R*) and (*S*) enantiomeric isoforms (following chiral separation) is a valid approach for modelling geographical use (Pérez-Alcaraz *et al.*, 2019).

Nonetheless, other studies have failed to detect mephedrone in urban cities of Norway (Reid *et al.*, 2014), Belgium (van Nuijs *et al.*, 2014; Bade *et al.*, 2017), Australia (Thai *et al.*, 2016), China (Khan *et al.*, 2014; Gao *et al.*, 2017b), Spain, Italy, Switzerland and The Netherlands (Bade *et al.*, 2017). The lack of detection of mephedrone (and methylone) in China is particularly surprising, given the reputation of China as a chief manufacturing base of synthetic cathinones (DEA, 2011), but may be indicative of differing patterns of recreational drug use relative to European countries: these same studies detected high levels of ketamine and methamphetamine, and low levels of MDMA/Ecstasy, cocaine and other NPS. However, it is also important to note that underestimations of mephedrone use might arise in studies which neglect to either: detect its metabolites (van Nuijs *et al.*, 2014); accommodate for potential confounds of data collection arising from sewage systems themselves (Ramin *et al.*, 2016); or appreciate the relative instability of mephedrone in some sewage systems and conditions (Reid *et al.*, 2014; McCall *et al.*, 2016; Gao *et al.*, 2017a; Ramin *et al.*, 2017; Kinyua *et al.*, 2018).

1.1.3.2 Context of recreational use

Like MDMA, mephedrone's consumption indicatively occurs mainly across the weekend (Castrignano *et al.*, 2016; Styszko *et al.*, 2016; Tscharke *et al.*, 2016; Bade *et al.*, 2017; Castrignano *et al.*, 2018), in keeping with its status as a drug used in the club/music/dance context. This is also indicated by data from gay-friendly night-clubs in London collected via survey (Wood *et al.*, 2012; Chan *et al.*, 2015) and amnesty bin sampling (Yamamoto *et al.*, 2013), as well as wastewater analyses of an Australian music festival (Lai *et al.*, 2013). As with most unregulated psychoactive substances, adulteration of products touted as mephedrone, and related derivatives, has been documented. Other synthetic cathinones are frequently employed for this purpose, and "bulking" is often conducted with cheap and readily available compounds such as caffeine, lidocaine, procaine, benzocaine, monosodium glutamate, creatine, sucrose, glutamic acid, ammonium sulfate and taurine (Brandt *et al.*, 2010a; Khreit *et al.*, 2012; Miserez *et al.*, 2014; Alotaibi *et al.*, 2015).

1.1.3.3 Contemporary use of mephedrone

Today, mephedrone use persists at a much reduced rate, relative to prior to international control (Vicknasingam *et al.*, 2020). In 2017, the EMCDDA reported that NPS use had declined since its 2010 peak (EMCDDA, 2017), whilst data elsewhere indicates mephedrone use to have decreased in the UK (Rice *et al.*, 2020). Nonetheless, mephedrone is described as being the only NPS stimulant to have become established in relation to conventional stimulants, with a similar number of emergency presentations, across Europe, to MDMA **(Fig. 1.7)** (EMCDDA, 2017), and an increase in police seizures between 2015 and 2017 (Vicknasingam *et al.*, 2020). Further, recent wastewater-based epidemiology data indicates mephedrone use to still occur in the UK (Castrignano *et al.*, 2018), and data from the Chinese Adolescents Health Survey show that between 2012/13 and 2016/17, mephedrone use continued at a steady rate (decreasing slightly from 0.30% lifetime use to 0.24% over this period) (Guo *et al.*, 2019).

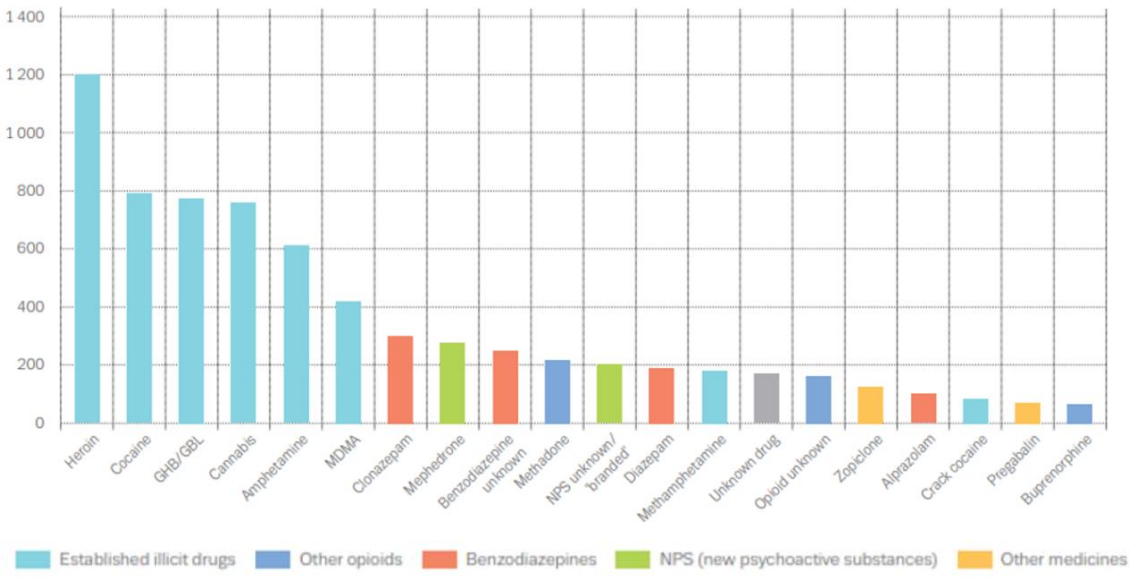


Figure 1.7 Mephedrone was identified in a similar number of emergency presentations to MDMA in selected European cities in 2015. Figure taken from EMCDDA (2017), and depicts the top 20 drugs recorded in 5,054 emergency presentations in 15 sentinel sites across nine European countries.

Although wastewater-based epidemiology data indicate a decline in mephedrone use in the UK between 2015 and 2018 (Rice *et al.*, 2020), data elsewhere indicate mephedrone remains in recreational use across Europe. In March 2019, for example, it was detected in wastewater samples of two Slovakian towns and of one city hosting an annual festival (Brandeburová *et al.*, 2020), as well as in wastewater pooled from eight different European locations (Celma *et al.*, 2019), and an oral fluid sample submitted anonymously from a private party in Europe (Bianchi *et al.*, 2019). Worryingly, between 2010 and 2018, a trend of increasing hospital admissions was noted in Warsaw, Poland, consequential of patients bingeing on mephedrone and engaging in polydrug use (Ordak *et al.*, 2018). During the COVID-19 global pandemic, shortages in the heroin market have led to the use of numerous substances as adulterants of heroin-based products, including synthetic opioids and synthetic cathinones (EMCDDA, 2020a), raising further concern for unknown drug interactions in these users.

1.1.3.4 Alcohol and mephedrone

Of monetarily incentivised "frequent" Ecstasy users (i.e. had consumed at least once a month for prior six months), alcohol and Ecstasy were co-consumed with energy drinks by 70 and 57% of respondents, respectively, in the preceding six months (Peacock *et al.*, 2016). Meanwhile, the co-consumption of alcohol (0.8 g kg^{-1}) with mephedrone (200.0 mg) has recently been shown to increase the cardiovascular effects of mephedrone in humans, whilst eliciting a more pronounced sensation of euphoria and well-being, relative to either drug alone (Papaseit *et al.*, 2019).

1.1.3.5 Use of mephedrone in sexual practices

In recent years, mephedrone has been popularly combined with methamphetamine and/or either gammahydroxybutyrate (GHB) or gamma butyrolactone (GBL) in the practice of chemical sex (or "chemsex"), most commonly amongst men who have sex

with men (MSM) (McCall *et al.*, 2015; Desai *et al.*, 2018; Edmundson *et al.*, 2018; Frankis *et al.*, 2018; Haugstvedt *et al.*, 2018; Melendez-Torres *et al.*, 2018; Barrett *et al.*, 2019; Hibbert *et al.*, 2019; Maxwell *et al.*, 2019; Sewell *et al.*, 2019; Busardò *et al.*, 2020).

In Germany, an online survey of methamphetamine-using MSM revealed 40.8% of participants had consumed mephedrone in a sexual setting (Schecke *et al.*, 2019). In London, a secondary analysis of Antidote (a UK-based specialist drug service for the LGBT community) data revealed that between January 2012 and June 2018, mephedrone was the most frequently mentioned drug (by 71% of respondents) by chemsex-practising MSM completing initial contact forms (Stevens *et al.*, 2020). As well as anecdotal evidence of the co-administration of mephedrone and GBL causing discolouration of the knees (perhaps due to a disruption of peripheral blood flow) (Assi *et al.*, 2017), chemsex-related deaths have been reported in the cases of a 26 year old male in Madrid (Troya *et al.*, 2019) and a 56 year old male in Parma (Anzillotti *et al.*, 2020). This is concerning, given that a study of used syringes conducted by the European Syringe Collection and Analysis Project Enterprise (ESCAPE) network found synthetic cathinones to account for 44 and 80% of contents in syringes recovered in Paris and Budapest, respectively, whilst mephedrone was the third most popular intravenously administered substance in seven locations across Hungary (Gyarmathy *et al.*, 2017), and its injection has been implicated in the development of Hepatitis C and HIV in UK recreational users (Hope *et al.*, 2016). Also, half of the syringes recovered during the study of five major European cities (also including Amsterdam, Glasgow and Helsinki) comprised a mixture of two or more drugs (most frequently a stimulant and opioid) (EMCDDA, 2019), whilst intra-arterial administration of mephedrone has been implicated in the onset of distal ischaemia in a 28 year old male's hand (Francés *et al.*, 2018). Further, the practice of chemsex appears to be evolving to incorporate the use of illicit opioids (Pirani *et al.*, 2019), rendering concerning speculation for currently unknown interaction effects mephedrone might exhibit when co-consumed with these drugs.

It is prudent to note that the literature concerning the use of mephedrone in a sexual context is disproportionately focused on the phenomenon of chemsex and the demographic of MSM. Alcohol (Malatesta *et al.*, 1982; Peugh & Belenko, 2001) and *Cannabis* (Halikas *et al.*, 1982) are known to increase sexual desire in both men and women, whilst sexual experiences have either been intentionally facilitated/prolonged or circumstantially improved, following the use of alcohol, cocaine, *Cannabis*, Ecstasy/MDMA, methamphetamine and opiates (Halikas *et al.*, 1982; Rawson *et al.*, 2002; Sumnall *et al.*, 2006; Bellis *et al.*, 2008; Palamar *et al.*, 2018). Future investigation of the use of sexualised mephedrone use would ideally focus on a broader demographic, accounting for a greater proportion of recreational users irrespective of sexual orientation or gender. This would assist in the enhanced illustration of mephedrone's contemporary recreational use, as well as lessening the potential for the discriminatory stereotyping of MSM.

1.1.3.6 Prescription drugs and mephedrone

The co-use of illicit compounds alongside clinically prescribed drugs is amongst the major concerns of clinicians. Adverse pharmacokinetic and pharmacodynamic interactions are of chief consideration in assessing the effects of such combinations. Amongst the potential issues is the capacity of some prescribed compounds to inhibit the metabolism of the illicit substance (de Leon & Nikoloff, 2008; Pedersen *et al.*, 2013; White, 2016). As a result of this inhibition, the antiarrhythmic drug quinidine and the antihistamine diphenhydramine have been shown to decrease the metabolism of mephedrone (White, 2016) and MDPV, respectively, the latter of which has been associated with at least one fatality (Kesha *et al.*, 2013). Elsewhere, a case has also been reported of death following combined use of synthetic cathinones alongside the atypical antipsychotic olanzapine and atypical antidepressant mirtrazapine (Liveri *et al.*, 2016). The co-use of mephedrone has also been documented in 8.6% of 93 drivers who were found to be driving following consumption of prescription drugs for a nonmedical purpose (Benotsch *et al.*, 2015), perhaps constituting a public safety concern for other road users. A further cause for concern, which will be described in

greater detail later in Chapter 1 (Section 1.4.1.2), is the co-use of mephedrone with selective serotonin reuptake inhibitors (SSRIs), which has the potential to increase the likelihood of adverse effects including the serotonin syndrome (Bartlett, 2017). Studies in this thesis will further examine the production of components of the serotonin syndrome by mephedrone alone and with caffeine.

1.1.3.7 Other illicit drugs and mephedrone

Of club-going MSM in New York City, sildenafil (often sold under the brand name Viagra®) was co-used at least once with methamphetamine (36.2% respondents), ketamine (26.2%), Ecstasy (25.0%), GHB (22.1%), and cocaine (18.9%); whilst other non-surveyed drugs, including alcohol, cannabis and heroin, were cited as having been used in conjunction (Halkitis & Green, 2007). In another group of self-reported recreational drug-using HIV-diagnosed MSM in the UK, most respondents admitted having used two (21%), three (16%), four (10%), five or more (21%) drugs within the past three months (Daskalopoulou *et al.*, 2014).

Similarly, data obtained from mephedrone users online (Carhart-Harris *et al.*, 2011; Winstock *et al.*, 2011b; Assi *et al.*, 2017), in emergency departments (Wood *et al.*, 2011), and post-mortem (Schifano *et al.*, 2012; Gerace *et al.*, 2014) collectively indicate these persons to be polydrug users. Interaction effects arising from concomitant administration of mephedrone with different drugs is an important concern.

1.1.3.8 Caffeine and mephedrone

Caffeine, a licit substance ubiquitous to the night club context, is found in such caffeinated energy drinks as Red Bull®. Its consumption alongside alcohol (Pennay *et al.*, 2015) and designer drugs, including Ecstasy and mephedrone, is indicated through

self-reports of recreational users, with reported elevations in mood (Assi *et al.*, 2017). Caffeine was also noted as a recurrent adulterant in needles collected in seven locations across Hungary, a third of which contained mephedrone (Gyarmathy *et al.*, 2017). Given caffeine's licit and cheap availability (Cole *et al.*, 2011; Broseus *et al.*, 2016) and indicated (Vidal Gine *et al.*, 2016) and verifiable (Brandt *et al.*, 2010a; Elie *et al.*, 2012; Zuba & Byrska, 2013) employment in adulteration, at any position of the distribution chain (Broseus *et al.*, 2016), it is reasonable to suggest caffeine as an adulterant in the sample collected by Gyarmathy *et al.* (2017).

A longitudinal study of donated Ecstasy products conducted between 2000 and 2014 indicates adulteration has increased rapidly since 2008/2009, with caffeine the most prevalent adulterant, present in around 30% of both tablets and crystals (Vidal Gine *et al.*, 2016). Similarly, caffeine and MDMA are common adulterants of mephedrone samples (EMCDDA, 2010b), whilst mephedrone and caffeine are also identified adulterants of amphetamine and Ecstasy products (Gine *et al.*, 2014; Hondebrink *et al.*, 2015). Given this widespread implication of caffeine as an adulterant and co-consumed substance alongside mephedrone, the remainder of this thesis will focus on the interaction between these two compounds.

1.1.4 Effects in humans

Most frequently administered orally or intranasally (Winstock *et al.*, 2011b), onset of mephedrone's effects typically occur 15–45 and 10–15 minutes post-administration, lasting for 2–5 and 2–3 hours, respectively (Karila & Reynaud, 2011). Desirable effects are described by users as those characteristic of stimulants, including elevations in alertness, mood, music appreciation and sensuality (Karila & Reynaud, 2011), though adverse effects both cognitive (anxiety and confusion) and physical (tachycardia, nausea, hypertension and seizure) are also commonly reported (Hill & Thomas, 2011; Karila & Reynaud, 2011; Winstock *et al.*, 2011b; Wood *et al.*, 2011; Assi *et al.*, 2017;

Papaseit *et al.*, 2017; Homman *et al.*, 2018). Case studies have also reported mephedrone-induced hyponatremia (Sammler *et al.*, 2010).

1.1.5 Effects in experimental animals

The use of rats in scientific research began around 1850, whilst the utilisation of the laboratory rat to model human processes both neuropsychological and neurobiological dates back at least a century (Jacob, 1999; Weiss & Feldon, 2001; Logan, 2005). The translational relevance of the rat as a model was greatly enhanced following the publication of the rat genome in 2004 (Gibbs *et al.*, 2004). As per the Animal (Scientific Procedures) Act 1986, in the absence of "a scientifically satisfactory alternative method or testing strategy not entailing the use of a protected animal", it is an ethical requirement to use the 'lowest' species possible in preclinical research. In the case of analysing the effects of drug administration on behavioural responses and activity of the central nervous system in a manner which allows inferences to be made of the effects of these drugs in human users, it is necessary to use mammals, as opposed to invertebrates or cell lines.

There are a number of advantages in the use of rats, instead of mice, in the study of drug use, abuse and addiction. Firstly, it is suggested by several studies that rats provide a more analogous representation of human drug abuse and addiction. For instance, although nicotine is readily self-administered by rats (Brennan *et al.*, 2015), this behaviour is more difficult to demonstrate in mice (Contet *et al.*, 2010; Parker *et al.*, 2014). In addition, rats have been shown to exhibit an alcohol deprivation effect, characterised by compulsive alcohol consumption over a period of three to four days, following repeated periods of alcohol deprivation (a behaviour likely analogous to "relapse" in human alcoholics), an effect which was present for just one day in mice and absent after multiple deprivation periods (Vengeliene *et al.*, 2014). Further to this, the effects of MDMA are mediated chiefly via dopamine (DA) in mice, and by serotonin (5-HT) in both rats (Kindlundh-Högberg *et al.*, 2007) and humans (Roberts *et al.*, 2016),

as indicated by reductions in drug-induced dopamine and 5-HT transporter (DAT and SERT, respectively) (Percie du Sert *et al.*, 2019) binding, respectively. A further indication of the relevance of 5-HT in the mediation of MDMA's effects in rats is evidenced following the deletion of SERT, which otherwise functions to remove 5-HT from the synapse via reuptake. Following genetic deletion of SERT, rats remain sensitive to the rewarding effects of MDMA (Oakly *et al.*, 2014), whereas no such effect is observed in mice (Trigo *et al.*, 2007).

A further advantage is that the physical size of the rat – and specifically the rat brain – relative to the mouse, renders it a more viable candidate for *in vivo* microdialysis research, such as that conducted in this thesis (Chapter 3). The use of a larger animal increases the sensitivity of this method by decreasing both the difficulty of stereotaxic surgery and the surface area potentially damaged by cannula implantation. Lastly, it is also easier to study the long-term effect(s) of a drug treatment on neurogenesis in rats than in mice. Data indicates the rate of adult hippocampal neurogenesis is faster in rats, maturing around two weeks earlier, and exhibiting around ten times the activity during learning (Snyder *et al.*, 2009). Collectively, these advantages posit rats as an optimal species for modelling human drug use, abuse and addiction (Parker *et al.*, 2014).

1.2 Neurochemistry

1.2.1 Mephedrone *in vitro*

In vitro assays can be used to determine the interaction of a drug with isolated components of a living organism, such as the pharmacological interaction at a specific receptor or receptor subtype. Noradrenaline (NET), dopamine (DAT) and serotonin transporters (SERT) are transmembrane proteins which regulate neurotransmission through the reuptake of monoamine neurotransmitters (MATs) (Kristensen *et al.*, 2011). Disruption of the function of these transporters is typically achieved either

through inhibition of their capacity to reuptake extracellular monoamines (such as in the case of cocaine), or through transportation of the substrate into the cell itself (such as the amphetamines).

In vitro preparations which have been used to study the pharmacological effects of mephedrone at these MATs include rat brain synaptosomes – membrane-bound sacs comprising synaptic vesicles, produced by homogenisation and subsequent centrifugation of brain tissue – as well as cells of the human embryonic kidney (HEK293) cell line.

1.2.1.1 Mephedrone as an uptake inhibitor

Uptake inhibitors (or transport blockers) such as cocaine and SSRIs prevent the reuptake of extracellular monoamines into the presynaptic neuron via MATs, rendering an increase in extracellular monoamine content, with differing levels of selectivity. Specifically, whilst SSRIs selectively prevent the reuptake of 5-HT, cocaine functions in a non-selective manner, preventing the reuptake of monoamines in general.

Like cocaine, mephedrone acts as a non-selective uptake inhibitor, exhibiting inhibition potencies (IC_{50}) in the micromolar to nanomolar range at DAT, SERT and NET of transfected HEK293 cells (Eshleman *et al.*, 2013; Simmler *et al.*, 2013; Pifl *et al.*, 2015; Mayer *et al.*, 2016), as well as inhibiting uptake via DAT, SERT (Hadlock *et al.*, 2011; Martinez-Clemente *et al.*, 2012) and NET (Lopez-Arnau *et al.*, 2012) in rat brain synaptosomes **(Table 1.1)**.

Table 1.1 Mephedrone has been shown to act as a reuptake inhibitor at the monoamine transporters. Effective concentrations of mephedrone at NET, DAT and SERT. Values are provided as half maximal inhibitory concentration (IC_{50}), to 2 decimal places, determined in the presence of a labelled inhibitor. Compound(s) specific to each study are provided in brackets. Data obtained from [1]Hadlock *et al.* (2011) (DAT: cocaine; SERT: fluoxetine), [2]Martinez-Clemente *et al.* (2012) (DAT: cocaine; SERT: fluoxetine), [3]Lopez-Arnau *et al.* (2012) (VMAT2: reserpine), [4]Eshleman *et al.* (2013) (hNET: [^{3}H]-NE; hDAT: [^{3}H]-DA; hSERT: [^{3}H]-5-HT), [5]Simmler *et al.* (2013) (hNET: *N*-methyl-[^{3}H]-nisoxetine & indatraline; hDAT: [^{3}H]-WIN35,428 and indatraline; hSERT: [^{3}H]-citalopram & indatraline), [6]Mayer *et al.* (2016) (hNET and hDAT: mazindol; hSERT: paroxetine), [7]Rickli *et al.* (2015) (hNET: nisoxetine; hDAT: mazindol; hSERT: fluoxetine), and [8]Pifl *et al.* (2015) (hNET and hDAT: mazindol; hSERT: fluoxetine). *HEK293 is a human embryonic kidney cell line, and the studies cited here probe the effect of mephedrone on HEK293 cells expressing the human recombinant transporters (hDAT, hSERT, hNET); **SK-N-MC is a human neuroblastoma cell line which originated from an Askin's tumour.

Cell culture	**Half maximal inhibitory concentration (IC_{50}) (µM)**				**NET:DAT ratio**	**DAT:SERT ratio**
	NET	**DAT**	**SERT**	**VMAT2**		
Rat brain synaptosomes		0.47 [1]	0.56 [1]			1.20
		0.97 [2]	0.31 [2]	3.40 [3]		0.32
	0.18 [3]					
*HEK293	0.05 [4]	0.10 [4]	0.51 [4]		1.83	5.20
	0.25 [5]	3.31 [5]	4.64 [5]		13.03	1.40
	2.77 [6]	0.77 [6]	7.83 [6]		0.28	10.17
	0.26 [7]	5.7 [7]	2.20 [7]		21.92	0.39
**SK-N-MC; HEK293	1.90 [8]	5.90 [8]	19.30 [8]		3.11	3.27

1.2.1.2 Mephedrone as a transporter substrate

Substrates of MATs, such as amphetamines, are actively transported into the cell before eliciting monoamine efflux across the plasma membrane via reverse transport of cytosolic monoamines (Sitte & Freissmuth, 2015).

In rat brain synaptosomes, mephedrone, like MDMA, has been shown to elicit the release of tritiated *N*-Methyl-4-phenylpyridinium ([^{3}H]-MPP+; a substrate for DAT, SERT and NET, as well as the organic cation ion transporter) and serotonin ([^{3}H]-5-HT) via DAT/NET and SERT respectively, characteristic of a substrate at these transporters (Baumann *et al.*, 2012), and consistent with prior demonstration of mephedrone-induced dopamine release from rat striatal suspension (Hadlock *et al.*, 2011). Similarly, mephedrone elicited transporter-mediated release of [^{3}H]-DA, [^{3}H]-5-HT and [^{3}H]-NE from HEK293 cells transfected with human DAT (hDAT), SERT (hSERT) and NET (hNET) respectively (Eshleman *et al.*, 2013; Simmler *et al.*, 2013), and evoked depolarisation in frog (*Xenopus laevis*) oocyte preparations transfected with human dopamine transporter (hDAT), characteristic of a dopamine-releasing agent (Cameron *et al.*, 2013a; Cameron *et al.*, 2013b) **(Table 1.2)**.

Once inside the cell, MDMA depletes vesicular storage of monoamines through the reversal of vesicular monoamine transporter 2 (VMAT2) activity (Capela *et al.*, 2009). Mephedrone has been shown to cause a concentration dependent inhibition of [^{3}H]-DA uptake at VMAT2 *in vitro* (Lopez-Arnau *et al.*, 2012) **(Table 1.1)** at a rate seven and 27 times more potent than methylone and butylone, respectively. However, the high micromolar range required for mephedrone binding to VMAT2 **(Table 1.3)** renders this an improbable function of mephedrone *in vivo*, unless perhaps administered at sufficiently high doses. An illustration of the dual mechanism of mephedrone as a transporter substrate and reuptake blocker is depicted in **Fig. 1.8**.

Table 1.2 Mephedrone has been shown to act as a substrate at the monoamine transporters. Effective concentrations of mephedrone at NET, DAT and SERT. Values are provided as half maximal effective concentration (EC_{50}), to two decimal places. Radiolabelled substrates specific to each study are provided in the "preloaded compound" column, with the exception of one study, in which EC50 was determined by the application of an inward current[5]. Data obtained from [1]Baumann *et al.* (2012), [2]Mayer *et al.* (2016), [3]Eshleman *et al.* (2013), [4]Simmler *et al.* (2013) and [5]Cameron *et al.* (2013a).

***In vitro* preparation**	**Preloaded compound**	**Half maximal effective concentration (EC_{50}) (μM)**			**NET:DAT ratio**	**DAT:SERT ratio**
		NET	**DAT**	**SERT**		
Rat brain synaptosomes	NET: [^{3}H]-MPP DAT: [^{3}H]-MPP SERT: [^{3}H]-5-HT	0.06 [1]	0.05 [1]	0.12 [1]	0.78	2.41
		0.09 [2]	0.05 [2]	0.21 [2]	0.58	4.04
HEK293	hNET: [^{3}H]-NE hDAT: [^{3}H]-DA hSERT: [^{3}H]-5-HT	0.41 [3]	1.19 [3]	11.90 [3]	2.90	6.26
			3.75 [4]	5.98 [4]		1.60
Xenopus laevis oocytes	hDAT [at -60 mV]		0.84 [5]			

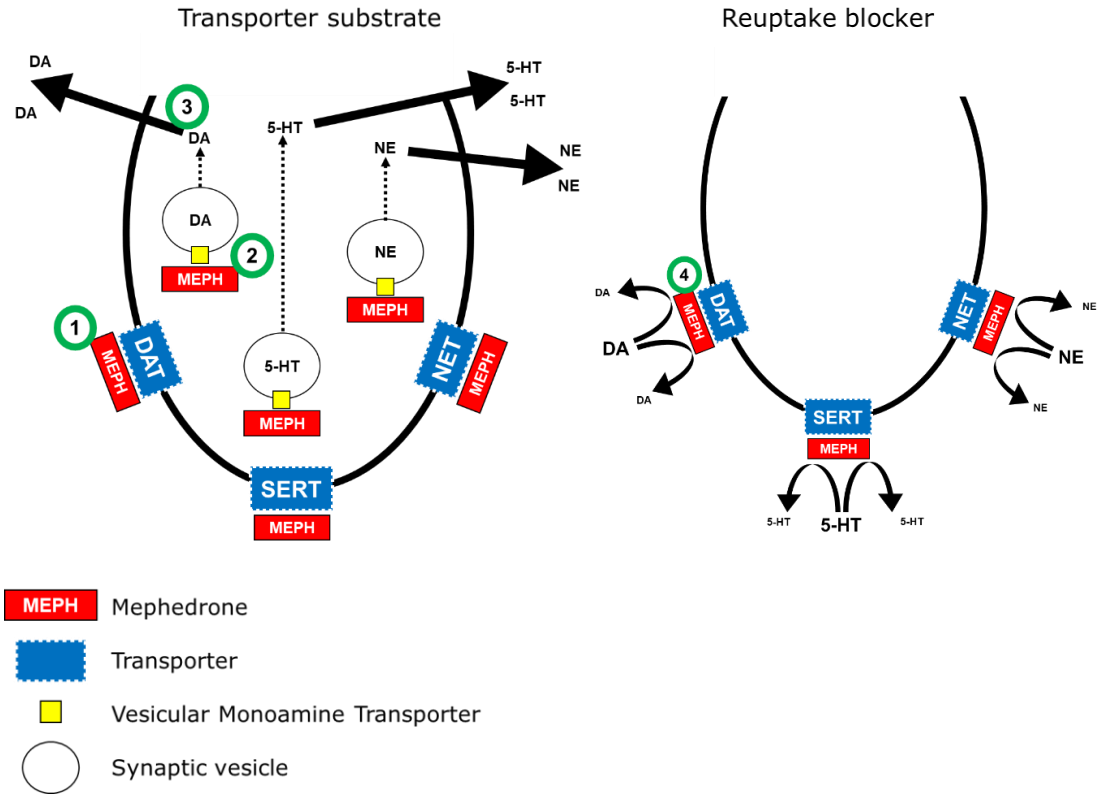


Figure 1.8 Mephedrone has been shown to elicit increases in synaptic monoamine content via a dual mechanism, functioning as a transporter substrate and reuptake blocker. Mephedrone enters the cell via transportation (by NET, DAT or SERT) **(1)** and disrupts vesicular storage **(2)**, causing transporter efflux **(3)**. Mephedrone also prevents the reuptake of monoamines into the cell via transporter blockade **(4).**

1.2.1.3 Binding affinity of mephedrone at receptors

In relation to its activity at other receptors, mephedrone exhibits its highest affinity (K_i < 10.0 µM) for α_{1A}-adrenoceptors, 5-$HT_{2A,}$ trace amine associated 1 (rat TA_1) and σ_1 (sigma-1) receptors, and lower affinity (K_i < 10.0 – 100.0 µM) for α_{2A}-adrenoceptors, 5-HT_{1A}, D_1, D_2 and histamine H_1 receptors, as well as mouse and human TA_1 (Lopez-Arnau *et al.*, 2012; Eshleman *et al.*, 2013; Simmler *et al.*, 2013). Mephedrone's affinity for 5-HT_{2C} receptors is less conclusive, with results differing in accordance with the radioligand used in this determination **(Table 1.3)**, perhaps as a result of the binding affinities and resultant effects of these compounds at other receptors.

Table 1.3 Mephedrone and MDMA have been shown to differ in their binding affinities for various transporters/receptors. Values provided as inhibitory constant K_i (µM). Data obtained from [1]Simmler *et al.* (2013), [2]Lopez-Arnau *et al.* (2012), [3]Eshleman *et al.* (2013) and [4]Rickli et al. (2015). In each study, specific binding of radioligands to the target receptors was determined by calculating the difference between total and nonspecific binding in the presence of specific competitors (MDMA or mephedrone).

Receptor/ transporter	Radioligand displaced	*In vitro* preparation	Inhibitory constant K_i (µM)	
			MDMA	Mephedrone
SERT	*[^{3}H]-citalopram*	HEK293	13.3 ± 0.6 [1]	> 30.0 [1]
	[^{3}H]-paroxetine	Rat cortex homogenates		17.55 ± 0.78 [2]
	[^{125}I]-RTI-55	HEK293	14.7 ± 2.9 [3]	21.0 ± 4.8 [3]
DAT	*[^{3}H]-WIN35428*	HEK293	6.5 ± 2.5 [1]	3.4 ± 0.8 [1]
	[^{3}H]-WIN35428	Rat striatal homogenates		1.53 ± 0.47 [2]
	[^{125}I]-RTI-55	HEK293	22.0 ± 5.1 [3]	4.80 ± 0.75 [3]
NET	*N-methyl-[^{3}H]-nisoxetine*	HEK293	30.5 ± 8.0 [1]	> 25.0 [1]
	[^{125}I]-RTI-55	HEK293	30.9 ± 5.6 [3]	11.8 ± 4.0 [3]
VMAT2	*[^{3}H]-DHTB*	HEK293	661 ± 58 [3]	> 1000.0 [3]
5-HT$_{1A}$	*[^{3}H]-8-OH-DPAT*	HEK293	12.2 ± 0.8 [1]	> 20.0 [1]
	[^{3}H]-8-OH-DPAT	HEK293	14.1 ± 2.4 [3]	> 91.0 [3]
5-HT$_{2A}$	*[^{3}H]-ketanserin*	HEK293	7.8 ± 2.4 [1]	2.1 ± 0.7 [1]
	[^{3}H]-ketanserin	Rat cortex homogenates		3.96 ± 0.22 [2]
	[^{125}I]-DOI	HEK293	8.3 ± 1.2 [3]	9.0 ± 1.5 [3]
5-HT$_{2C}$	*[^{3}H]-mesulergine*	HEK293	> 13.0 [1]	> 13.0 [1]
	[^{125}I]-DOI	HEK293	1.36 ± 0.44 [3]	9.1 ± 2.1 [3]
α_{1A}	*[^{3}H]-prazosin*	HEK293	> 6.0 [1]	3.48 ± 2.2 [1]
α_{2A}	*[^{3}H]-rauwolscine*	HEK293	15.0 ± 10.0 [1]	11.0 ± 5.0 [1]

D_1	*[^{3}H]-SCH23390*	HEK293	> 13.6 [1]	> 13.6 [1]
D_2	*[^{3}H]-spiperone*	HEK293	25.2 ± 12 [1]	> 30.0 [1]
	[^{3}H]-raclopride	Rat striatal homogenates		50.86 ± 3.45 [2]
D_3	*[^{3}H]-spiperone*	HEK293	> 17.7 [1]	> 9.2 [1]
H_1	*[^{3}H]-pyrilamine*	HEK293	> 14.4 [1]	> 14.4 [1]
TA_1Rat	*[^{3}H]-RO5166017*	HEK293	0.37 ± 0.12 [1]	4.3 ± 2.0 [1]
TA_1Mouse	*[^{3}H]-RO5166017*	HEK293	2.4 ± 1.1 [1]	> 10.0 [1]
TA_1Human	*[^{3}H]-RO5166017*	HEK293	14.6 ± 1.8 [4]	> 20.0 [4]
σ_1	*[^{3}H]-pentazocine*	HEK293	19.4 ± 6.3 [3]	7.8 ± 2.0 [3]

1.2.2 Mephedrone *in vivo*

1.2.2.1 Acute effects on brain monoamines

In vivo microdialysis is a technique in which extracellular fluid content of a living brain can be sampled for neurochemical analysis. This fluid, comprising unbound analytes (including dopamine, 5-HT, and their major metabolites) can be collected via the semipermeable membrane of a microdialysis probe from the synaptic overflow of specific brain regions of conscious, freely-moving rodents. The monoamines and amino acids present can then be separated, identified and quantified by high performance liquid chromatography with electrochemical detection (HPLC-ED) (Krebs-Kraft *et al.*, 2007). Since the 1990s, amino acids have been more commonly quantified by mass spectrometry or fluorescence following conjugation (Millington *et al.*, 1991).

Consistent with the findings of *in vitro* assays, mephedrone elicits a dose-dependent elevation of extracellular dopamine and 5-HT content in nucleus accumbens (NAcc) (Kehr *et al.*, 2011; Baumann *et al.*, 2012; Wright *et al.*, 2012; Golembiowska *et al.*, 2016; Mayer *et al.*, 2016; Suyama *et al.*, 2016; Lopez-Arnau *et al.*, 2018) **(Table 1.4)**, striatum (Golembiowska *et al.*, 2016; Shortall *et al.*, 2016b; Lopez-Arnau *et al.*, 2018)

and frontal cortex (Golembiowska *et al.*, 2016; Lopez-Arnau *et al.*, 2018) of rats. Similar elevations of dopamine have been observed in the NAcc, ventral tegmental area (VTA), striatum and substantia nigra of Swiss-Webster mice (Allen *et al.*, 2019). Despite mephedrone's greater action *in vitro* as a substrate and blocker at DAT relative to SERT, these *in vivo* rat data indicate preferential elevation in all areas of 5-HT relative to dopamine **(Table 1.4)**.

The rapidity of these increases is largely contingent on mephedrone's route of administration, with increases observed following intravenous (i.v.) and subcutaneous (s.c.) injection (within 15 to 20 minutes) than by intraperitoneal (i.p.) (within 30 minutes) **(Table 1.4)**. Irrespective of administration route, dopamine and 5-HT levels generally peak around 40 min post-administration of mephedrone, before returning to basal levels within one hour following low doses (0.3 – 3.2 mg kg^{-1}) and two to three hours following high doses (10.0 – 25.0 mg kg^{-1}), consistent with user self-reports of psychoactive effects within this time frame (Schifano *et al.*, 2011; Winstock *et al.*, 2011b).

Mimicking the human practice of re-dosing, some studies have measured the effect of repeated administration of mephedrone *in vivo*. In a regimen in which rats were administered mephedrone (10.0 mg kg^{-1}, i.p.) thrice across six hours (i.e. two hours between each injection), Shortall *et al.* observed that extracellular striatal dopamine and 5-HT content peaked around 40 minutes and returned to baseline between each injection (Shortall *et al.*, 2016b), akin to striatal effluxes observed following repeated administration of MDMA (Green *et al.*, 2003), and consistent with psychoactive experiences cited by human mephedrone users.

1.2.2.2 Acute effects of co-administration of mephedrone and other drugs

Given the co-use of mephedrone with other licit and illicit drugs, it is prudent that *in vivo* methods are used to assess the effects of these additional substances on mephedrone-induced changes in neurochemistry.

Mephedrone's capacity to affect monoamine effluxes is augmented through co-administration of other substances. Ethanol has been shown to elongate the elevation time of mephedrone-induced dopamine and 5-HT accumbal dialysate efflux in Sprague-Dawley rats – perhaps via inhibition of GABA-ergic interneurons terminating in this area (Siggins *et al.*, 2005) – as well as of 5-HT in medial prefrontal cortex (mPFC) (Lopez-Arnau *et al.*, 2018), whilst fluoxetine has been shown to prevent mephedrone-induced SERT-mediated [^{3}H]-MPP$^+$ efflux in HEK293 cells *in vitro* (Pifl *et al.*, 2015). Importantly, although this latter finding indicates that SSRIs may prevent mephedrone's capacity to act as a reuptake inhibitor, it does not rule out its ability to effect monoamine efflux via its action as a transporter substrate. Consequentially, the co-use of mephedrone and SSRIs might constitute a hazardous interaction whereby synaptic 5-HT content is greatly enhanced relative to following either drug alone, raising the possibility of the serotonin syndrome (Bartlett, 2017).

In mice, co-administration with other bath salt constituents (MDPV and methylone) (3.3 mg kg^{-1} each, i.p.) potentiated mephedrone-induced increases in dopamine content of NAcc, striatum, VTA and substantia nigra, 15 minutes following administration (Allen *et al.*, 2019), indicating an additive or synergistic effect which raises concern for the abuse or addictive potential of these drugs when co-administered by humans.

Table 1.4 Mephedrone has been shown to cause an increase in extracellular dopamine and 5-HT content in rat NAcc. Times of dopamine and 5-HT increases appear contingent on route of administration to Sprague-Dawley (SD) or Wistar (W) rats, with faster increases observed following intravenous (i.v.) and subcutaneous (s.c.) administration, relative to intraperitoneal (i.p.). Where elevation times are not included, this is due to a lack of clarification *re* specific timeframes of elevation in the publication cited. *approximate graphical values not clarified in publication cited. Data obtained from [1]Baumann *et al.* (2012), [2]Mayer *et al.* (2016), [3]Kehr *et al.* (2011), [4]Suyama *et al.* (2016), [5]Golembiowska *et al.* (2016), [6]Wright *et al.* (2012) and [7]Lopez-Arnau *et al.* (2018).

Dose (mg kg^{-1})	Route of admin.	Rat strain	DA		5-HT	
			Elevation time (mins post-injection)	Peak basal value (peak time)	Elevation time (mins post-injection)	Peak basal value (peak time)
0.3 [1]	i.v.	SD	20-40	180% (20)	20	420% (20)
1.0 [1]			20-60	290% (20)	20-60	1100% (20)
1.0 [2]			20-40	~275% (20)	20-40	~156% (20)
1.0 [3]	s.c.		20	295% (20)	20-40	709%
1.0 [4]	i.p.		70-100	~150% (80)*	30-40, 160-180	~200% (30)*
3.0 [3]	s.c.		20-60	496% (40)	20-40	941% (20)
3.2 [4]	i.p.	SD	70-150, 170-180	~250% (70)*	30-50	~750% (30)*
5.0 [5]		W		~150% (20)		~290% (100)
10.0 [4]		SD	50-180	~500% (80)*	30-80	~1100% (30)*
10.0 [5]		W		~190% (20)		~380% (100)
10.0 [6]	s.c.	SD	15-120	900% (30)	15-90	2200% (30)
20.0 [5]	i.p.	W		~300% (140)		~550% (40)
25.0 [7]	s.c.	W	20-60	~5000% (40)	20-120	~9000% (40)

1.2.2.3 Pharmacological activity of mephedrone's metabolites

Interestingly, nor-mephedrone and 4-OH-mephedrone, two phase I metabolites of mephedrone, have been shown to function in a manner akin to mephedrone itself,

acting both as potent substrates **(Table 1.5)** and relatively weak uptake inhibitors **(Table 1.6)** at monoamine transporters in HEK293 cells and rat brain synaptosomes (Mayer *et al.*, 2016). Relative to mephedrone itself, nor-mephedrone exhibited equipotent transporter-mediated efflux via both NET and SERT, and a four-fold lower potency at DAT, in rat brain synaptosomes (Mayer *et al.*, 2016). The (*S*)-enantiomer of nor-mephedrone, the form in which the metabolite predominantly exists according to chiral analysis of human urine samples, is more than twenty-fold more potent than (*R*)-nor-mephedrone as a substrate (EC_{50} = 10.88 and 200.0 µM, respectively) (Mayer *et al.*, 2019).

The pharmacological activity of 4-OH-mephedrone is comparatively less, exhibiting two-fold and four-fold lower potency as a transporter substrate at NET and DAT in rat brain synaptosomes, respectively, and a ten-fold lower potency at SERT (Mayer *et al.*, 2016). Further, unlike nor-mephedrone, 4-OH-mephedrone failed to cause elevations in either locomotor activity or extracellular dopamine and 5-HT in rats *in vivo* (Mayer *et al.*, 2016).

Nonetheless, compounded with: the detection of 4-OH-mephedrone in human blood plasma up to six or ten hours following, respectively, nasal (Steuer *et al.*, 2020) or oral (Papaseit *et al.*, 2019) administration of mephedrone (100.0 or 200.0 mg, respectively) (Steuer *et al.*, 2020); as well as nor-mephedrone in rat blood serum, brain, lungs and liver seven to eight hours post-administration (Sichova *et al.*, 2017); these data raise the possibility of additive effect of these metabolites on the effects of mephedrone, particularly in the practice of rapid re-dosing observed in humans.

Recently, Řezanka *et al.* developed a capillary electrophoresis method to allow the chiral separation of mephedrone (and its metabolites) into (*R*) and (*S*) enantiomeric isoforms (Řezanka *et al.*, 2020). Techniques such as this allow more accurate characterisation of the neurochemical effects of (*R*)-mephedrone and (*S*)-

mephedrone, individually, as well as pharmacologically active metabolites of mephedrone. For instance, urine samples indicate that nor-mephedrone exists mainly in its (*S*)-enantiomer form, which exhibits several-fold greater affinity as a SERT reuptake blocker than its (*R*)-enantiomer, potentially causing a synergistic effect on synaptic 5-HT content in users re-dosing mephedrone (Mayer *et al.*, 2019).

Table 1.5 Some Phase I metabolites of mephedrone have been found to act as substrates of the monoamine transporters. Effective concentrations of metabolites of mephedrone at NET, DAT and SERT in rat brain synaptosomes. Values are provided as half maximal effective concentration (EC_{50}). Data obtained from Mayer *et al.* (2016).

Metabolite	**Preloaded compound**	**Half maximal effective concentration (EC_{50}) (μM)**			**NET:DAT ratio**	**DAT:SERT ratio**
		NET	**DAT**	**SERT**		
Nor-mephedrone	NET: [^{3}H]-MPP$^+$ DAT: [^{3}H]-MPP$^+$ SERT: [^{3}H]-5-HT	0.1 (0.08-0.13)	0.22 (0.14-0.32)	0.21 (0.13-0.32)	2.20	0.96
4-OH-mephedrone		0.15 (0.11-0.19)	0.19 (0.13-0.267)	2.01 (1.390-2.91)	1.27	10.58

Table 1.6 Some Phase I metabolites of mephedrone demonstrate efficacy as reuptake inhibitors at the monoamine transporters. Effective concentrations of metabolites of mephedrone at NET, DAT and SERT in HEK293 cells. Values are provided as half maximal inhibitory concentration (IC_{50}), to two decimal places. Data obtained from [1]Mayer *et al.* (2016) and [2]Mayer *et al.* (2019).

Metabolite	**Half maximal inhibitory concentration (IC_{50}) (μM)**			**NET:DAT ratio**	**DAT:SERT ratio**
	NET	**DAT**	**SERT**		
Nor-meph [1]	5.46 (3.58-8.31)	6.35 (4.66-8.64)	10.61 (9.06-12.43)	1.16	1.67
S-nor-meph [2]	3.84 (3.16-4.66)	8.72 (5.9-12.87)	10.88 (6.33-18.7)	2.27	1.25
R-nor-meph [2]	4.54 (3.44-5.99)	9.44 (6.51-13.68)	200.0 (106.8-374.6)	2.08	21.19
4-OH-meph [1]	4.85 (3.28-7.17)	2.92 (2.35-3.6)	73.53 (62.5-86.51)	0.60	25.18
S-4-OH-meph [2]	9.53 (7.49-12.14)	2.63 (1.98-3.49)	32.05 (24.39-42.13)	0.28	12.19
R-4-OH-meph [2]	18.04 (11.76-27.67)	5.64 (3.68-8.64)	625.40 (478-818.3)	0.31	110.89
Dihydromephedrone [1]	23.53 (19.8-27.97)	23.97 (8.65-66.46)	64.98 (50.66-83.37)	1.02	2.71

1.2.2.4 Long-term effect of MDMA on brain monoamines

In rats, MDMA is evidenced to effect lasting neurotoxicity on serotonergic neurons (Commins *et al.*, 1987; Schmidt, 1987) and in multiple brain regions following repeated or high dose administration (Stone *et al.*, 1986; Battaglia *et al.*, 1987) **(Fig. 1.9)**. Such effects are most pronounced in striatum, hippocampus and frontal cortex, and are observed in tandem with lasting decreases in tryptophan hydroxylase (TPH) activity, as well as reductions in [^{3}H]-paroxetine binding and uptake at SERT (O'Shea *et al.*, 2006; Xie *et al.*, 2006). Beginning between 24 hours and seven days following administration, these depletions are independent of the MDMA-induced 5-HT release observed within 24 hours of administration (Schmidt & Taylor, 1987).

Importantly, this neurotoxicity in rats does not appear to result directly from MDMA itself, as indicated around 30 years ago following direct administration to the brain (Paris & Cunningham, 1992; Esteban *et al.*, 2001). Although not fully understood, it appears to result from the peripheral metabolism of MDMA, induction of free radicals (Aguirre *et al.*, 1999; Green *et al.*, 2003; Darvesh *et al.*, 2005), and consequential oxidative stress. Indeed the primary metabolite MDA is evidenced, like MDMA, to produce both acute hyperthermia and neurotoxic damage (Colado *et al.*, 1995; Green *et al.*, 2003). Specifically, it is suggested that via oxidation, the catechol metabolites of MDMA lose electrons to generate quinone metabolites, thereby increasing the production of free radicals, reactive oxygen (ROS) and reactive nitrogen species (RNS) (Capela *et al.*, 2009; Song *et al.*, 2010) **(Fig. 1.9)**. Importantly, the extent of MDMA metabolism (Goni-Allo *et al.*, 2008), free radical production (Colado *et al.*, 1997) and neurotoxic effect is demonstrably contingent on the body temperature of the rat following MDMA administration (Colado *et al.*, 1998), which can be increased by administration at higher room temperatures (i.e. between 28.0 and 30.0 °C) (Malberg & Seiden, 1998; Green *et al.*, 2004).

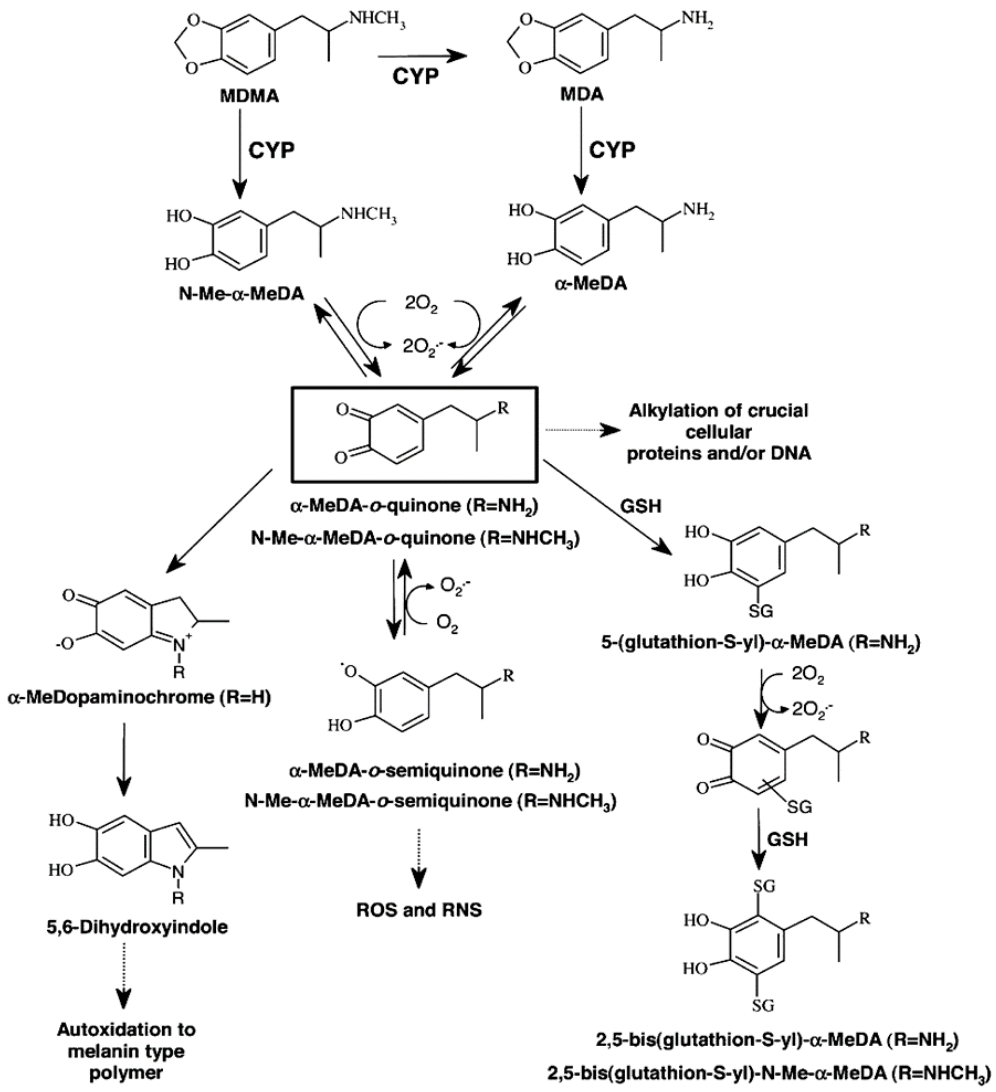


Figure 1.9 Production of MDMAs neurotoxic metabolites. MDMA is metabolised to MDA via *N*-demethylation. MDMA and MDA are metabolised, respectively, to N-Me-α-MeDa and α-MeDa, by cytochrome P450 (CYP) enzymes. Catechols of MDMA are oxidised to their corresponding *o*-quinones, which may then enter redox cycles with their corresponding semiquinone radicals, causing the formation of reactive oxygen (ROS) and reactive nitrogen species (RNS). Radical cyclisation of these *o*-quinones causes production of aminochromes (e.g. α-Medopaminochrome) and related compounds (e.g. 5,6-dihydroxyindoles). These compounds may be further oxidised and polymerised, forming black or brown insoluble pigments. Also, *o*-quinones may react with glutathione, forming corresponding glutathione conjugates (e.g. 5-GSH-α-MeDa). Figure taken from Green *et al.* (2012), reproduced from Capela *et al.* (2006).

Nonetheless, it remains problematic to translate the effect of MDMA in rats to humans, or indeed to other rodents. Although neurotoxic in mice, monoamine depletions appear to be exclusively dopaminergic, with long-term serotonergic measures unaffected by MDMA (Stone *et al.*, 1987; Logan *et al.*, 1988; Colado *et al.*, 2001; O'Shea *et al.*, 2001). This is suggested to be because MDMA is metabolised differently in mice (Colado *et al.*, 2001; Colado *et al.*, 2004; de la Torre & Farré, 2004; Escobedo *et al.*, 2005; Easton & Marsden, 2006; de la Torre *et al.*, 2009). In humans, however, the metabolic rate of MDMA is demonstrably slower, suggesting that the neurotoxic effects observed in rodents may be less problematic for human users, although contextual and polydrug interactions, such as the co-consumption of licit and illicit drugs in the nightclub/dance context, may alter the capacity of MDMA to elicit neurotoxicity (Green *et al.*, 2012).

1.2.2.5 Long-term effect of mephedrone on rat brain in vivo

Data concerning mephedrone's neurotoxic capacity at serotonergic **(Table 1.7)** and dopaminergic **(Table 1.8)** neurons is inconclusive and warrants further investigation. In Sprague-Dawley rats, mephedrone appears to selectively deplete 5-HT, relative to DA, in a wide range of brain areas three (Motbey *et al.*, 2013) and seven days post-administration (Hadlock *et al.*, 2011; Lopez-Arnau *et al.*, 2015), with no such depletions noted in either Sprague-Dawley or Wistar rats at two weeks (Baumann *et al.*, 2012; den Hollander *et al.*, 2013). Findings of monoamine deficits in these strains, and not in Lister-Hooded rats (Shortall *et al.*, 2013b; Shortall *et al.*, 2016a), might simply be attributable to variations in room temperature between studies, rather than strain-related differences.

In studies utilising the repeated administration of mephedrone across multiple days, mimicking the recreational use of human users, serotonergic deficits including depletion of 5-HT content and decreased [^{3}H]-paroxetine binding and activity at SERT, were noted in rats one week following repeated administration at high (> 26.0 ^{o}C)

(Hadlock *et al.*, 2011; Lopez-Arnau *et al.*, 2015) but not low (< 23.0 ^{o}C) ambient room temperature (den Hollander *et al.*, 2013; Shortall *et al.*, 2016b). Inconsistent effects were observed on dopaminergic measures following administration at high temperatures. Whilst repeated administration precipitated a depletion of tyrosine hydroxylase (TH) content in both striatum and frontal cortex, decreased DAT binding occurred only in frontal cortex (Lopez-Arnau *et al.*, 2015), with no effect on striatal dopamine content following a similar regimen (Hadlock *et al.*, 2011).

Likewise in mice, serotonergic deficits including depletion of SERT in frontal cortex and striatum, as well as decreased tryptophan hydroxylase 2 (TPH2) content, were observed one week following administration at high room temperature (Martinez-Clemente *et al.*, 2014; Ciudad-Roberts *et al.*, 2016b), whilst decreased DAT binding has been noted in frontal cortex and striatum between three and seven days following binge dosing regimens (Martinez-Clemente *et al.*, 2014; Ciudad-Roberts *et al.*, 2016b).

Lipid peroxidation, indicative of oxidative damage to lipids and cellular damage, has been observed in frontal cortex, but not striatum or hippocampus, of Sprague-Dawley rats six and 24 h following repeated administration of mephedrone (3 x 25.0 mg kg^{-1}, s.c., two hours apart, two consecutive days). Nonetheless, this was followed seven days post-mephedrone by SERT and TPH2 depletions in all three areas, as well as DAT and TH depletions in frontal cortex (Lopez-Arnau *et al.*, 2015). Interestingly, regional differences in lipid peroxidation have been observed in the effect of mephedrone in mice. Twenty four hours following a similar dosing regimen (4 x 25.0 mg kg^{-1}, s.c., two hours apart, two consecutive days), mephedrone elicited an increase in lipid peroxidation in the hippocampus, but not frontal cortex, of Swiss CD-1 mice (Ciudad-Roberts *et al.*, 2016b). It is at this time point that mephedrone has also been shown to precipitate an upregulation in genes relating to mitochondrial function, metabolic processes, gliosis, oxidative stress and apoptosis (Ciudad-Roberts *et al.*, 2015). However, consistent with the rat data of Lopez-Arnau *et al.* (2015), depletions in serotonergic (SERT, TPH2) and dopaminergic (DAT, TH) measures in hippocampus and

frontal cortex, respectively, were observed seven days post-administration, as well as impaired neurogenesis in the dentate gyrus at 28 days (Ciudad-Roberts *et al.*, 2016a). Collectively, these data indicate a region-specific susceptibility to mephedrone-induced oxidative stress, and an action of mephedrone to impair hippocampal neurogenesis in mice.

Methcathinone, another structural derivative of cathinone, rendered decreases in striatal DA, DAT and TH (tyrosine hydroxylase) content, accompanied by enhanced astroglial activation, in C57BL/6 mice two days following repeated administration (four injections, two hours apart) (Anneken *et al.*, 2017b). Increases in microglial and astroglial activation (indicative of neuronal damage) were not observed in this strain two days following administration of mephedrone in the same regimen, nor were depletions of DA, DAT or TH in striatum (Angoa-Perez *et al.*, 2012) or of 5-HT, 5-HIAA, SERT or TPH2 in hippocampus (Angoa-Perez *et al.*, 2014). The effect of mephedrone on both microglial and astroglial activation is yet to be examined in rats, but remains an area of concern in light of recent evidence of oxidative damage to cortical nuclei in adult rats as a result of exposure to mephedrone during adolescence (Kaminska *et al.*, 2018), as well as rapid (as early as five minutes in some measures, and by 60 minutes in all) mephedrone-induced oxidative stress and mitochondrial dysfunction, including increases in ROS production and cytochrome-c release (indicative of mitochondrial apoptosis) in hippocampus, cortex and cerebellum (Naserzadeh *et al.*, 2019).

Table 1.7 Long-term effect of mephedrone on serotonergic measures. Mephedrone has been evidenced to produce no consistent long-term effect on serotonergic measures in rats or mice. Table depicts the effect of mephedrone, administered at various doses and regimens, on measures of 5-HT, SERT, 5-HIAA, tryptophan hydroxylase 2 (TPH2), and the metabolic rate of 5-HT (the ratio of 5-HIAA/5-HT was used as an index for this measure). Brain regions investigated include cortex (CX), dorsal raphe (DR), frontal cortex (FCX), hippocampus (HIP), hypothalamus (HYP), olfactory tubercle (OT), nucleus accumbens shell (NAcc), striatum (STR), substantia nigra (SN) and ventral tegmental area (VTA); *indicates increased phosphorylation; [#]detail provided by author. Data obtained from [1]Motbey *et al.* (2013), [2]Shortall *et al.* (2013), [3]Shortall *et al.* (2016), [4]Hadlock *et al.* (2011), [5]Lopez-Arnau *et al.* (2015), [6]Baumann *et al.* (2012), [7]Den Hollander *et al.* (2013), [8]Kaminska *et al.* (2018), [9]Angoa-Perez *et al.* (2014), [10]Martinez-Clemente *et al.* (2014) and [11]Ciudad-Roberts *et al.* (2016).

Dosing, regimen	Species, strain	Room temp. (°C)	Brain area (s)	Time point of effect	Effect on…				
					5-HT	SERT	5-HIAA	TPH2	5-HT metabolism
0.3 – 1.0 mg kg^{-1} (i.v.), self-admin. [1]	Rat, SD	Not provided	STR, DR, HIP, OT, SN, VTA	+3 days	None (all)		(STR) (↓)		None (all)
4.0 or 10.0 mg kg^{-1} (i.p.), 2 consecutive days a week, 3 weeks [2]	Rat, LH		HIP	+7 days	None (all)		None (all)		
10.0 mg kg^{-1} (i.p.), 2 consecutive days a week, 3 weeks [3]		19.6 (±0.3)	HYP, STR, FCX, HIP		None (all)				
4 x 10.0 or 25.0 mg kg^{-1} (s.c.), 2 h apart [4]	Rat, SD	27.0	HIP		Content (all) (↓)	Activity (all) (↓)			
4 x 25.0 mg kg^{-1} (s.c.), 2 h apart, 2 consecutive days [5]		~26.0	STR, FCX, HIP			Binding (all) (↓)		Expression (all) (↓)	
3 x 10.0 mg kg^{-1} (s.c.), 2 h apart [6]		# low ambient	CX, STR	+14 days	None (all)				
2 x 30.0 mg kg^{-1} (i.p.), 2 consecutive days [7]	Rat, W	20.0 – 23.0	NAcc, STR, FCX		None (all)	None (all)	None (all)		

Dosing, regimen	Species, strain	Room temp. (°C)	Brain area (s)	Time point of effect	Effect on…				
					5-HT	SERT	5-HIAA	TPH2	5-HT metabolism
5.0 mg kg^{-1} (i.p.), 4 consecutive days, 3 days break, 4 consecutive days[8]	Rat, WH	#room temp.	STR, NAcc, FCX	+50 days	(STR, NAcc) (↓)		(STR, NAcc) (↓)		None (all)
4 x 20.0 mg kg^{-1} (i.p.), 2 h apart [9]	Mouse, C57BL/6	Not provided	HIP	+2 days	None (all)	None (all)	None (all)	None (all)	
4 x 25.0 mg kg^{-1} (s.c.), 2 h apart [10]	Mouse, Swiss CD-1	26.0 (±2.0)	HIP, FCX	+3 days		None (all)			
3 x 25.0 mg kg^{-1} (s.c.), 2 h apart [10]						(All) (↓)			
4 x 50.0 mg kg^{-1} (s.c.), 2 h apart [10]						(All) (↓)			
4 x 25.0 mg kg^{-1} (i.p.), 2 h apart [11]				+7 days		(HIP) (↓)		(HIP) (↓)	
4 x 25.0 mg kg^{-1} (s.c.), 2 h apart [10]						None (all)			

Dosing, regimen	Species, strain	Room temp. (°C)	Brain area (s)	Time point of effect	Effect on…				
					5-HT	SERT	5-HIAA	TPH2	5-HT metabolism
4 x 50.0 mg kg^{-1} (s.c.), 2 h apart [10]	Mouse, Swiss CD-1	26.0 (±2.0)	HIP, FCX	+7 days		None (all)			
3 x 25.0 mg kg^{-1} (s.c.), 2 h apart [10]						(All) (↓)		(All) (↓) *	
2 x 30.0 mg kg^{-1} (i.p.), 2 consecutive days [7]					None	None (all)	None (all)		

Table 1.8 Long term effect of mephedrone on dopaminergic measures. Mephedrone has previously been shown to produce no consistent long-term effect on dopaminergic measures in rats or mice. Table depicts the effect of mephedrone, administered at various doses and regimens, on measures of dopamine (DA), DAT, HVA, DOPAC, tyrosine hydroxylase (TH) and the metabolic rate of dopamine (the ratio of HVA+DOPAC/DA was used as an index for this measure). Brain regions investigated include cortex (CX), frontal cortex (FCX), hippocampus (HIP), hypothalamus (HYP), nucleus accumbens shell (NAcc), striatum (STR) and ventral tegmental area (VTA); [#]detail provided by author. Data obtained from [1]Motbey *et al.* (2013), [2]Shortall *et al.* (2013), [3]Shortall *et al.* (2016), [4]Hadlock *et al.* (2011), [5]Lopez-Arnau *et al.* (2015), [6]Baumann *et al.* (2012), [7]Den Hollander *et al.* (2014), [8]Kaminska *et al.* (2018), [9]Angoa-Perez *et al.* (2012), [10]Anneken *et al.* (2013), [11]Ciudad-Roberts *et al.* (2016) and [12]Martinez-Clemente *et al.* (2014).

Dosing, regimen	Species, strain	Room temp. (°C)	Brain area (s)	Time point of effect	Effect on…				
					DA	DAT	DOPAC or HVA	TH	DA metabolism
0.3 – 1.0 mg kg^{-1} (i.v.), self-admin.[1]	Rat, SD	Not provided	NAcc, SN, STR, VTA	+3 days	None (all)	None (all)	None (all)		None (all)
4.0 mg kg^{-1} (i.p.), 2 consecutive days a week, 3 weeks[2]	Rat, LH		HIP	+7 days	None (all)		DOPAC (↑)		
10.0 mg kg^{-1} (i.p.), 2 consecutive days a week, 3 weeks[2]					None (all)		DOPAC (↓)		
10.0 mg kg^{-1} (i.p.), 2 consecutive days a week, 3 weeks[3]		19.6 (±0.3)	HYP, STR, FCX, HIP		None (all)				
4 x 10.0 or 25.0 mg kg^{-1} (s.c.), 2 h apart[4]	Rat, SD	27.0	STR		None (all)				
4 x 25.0 mg kg^{-1} (s.c.), 2 h apart, 2 consecutive days[5]		~26.0	FCX, STR			Binding (FCX) (↓)		(↓)	
3 x 10.0 mg kg^{-1} (s.c.), 2 h apart[6]		#Low ambient	CX, STR	+14 days	None (all)				
2 x 30.0 mg kg^{-1} (i.p.), 4 consecutive days[7]	Rat, W	20.0 – 23.0	FCX, HIP, STR		None (all)	None (all)	None (all)		

Dosing, regimen	Species, strain	Room temp. (°C)	Brain area (s)	Time point of effect	Effect on…				
					DA	DAT	DOPAC or HVA	TH	DA metabolism
5.0 mg kg^{-1} (i.p.), 4 consecutive days, 3 days break, 4 consecutive days[8]	Rat, WH	#Room temp.	STR, NAcc, FCX	+50 days	(STR) (↑)		DOPAC (STR, FCX) (↑)		(STR, NAcc) (↓)
4 x 20.0 mg kg^{-1} (i.p.), 2 h apart[9]	Mouse, C57BL/6	Not provided	STR	+2 days	None (all)	None (all)		None (all)	
4 x 40.0 mg kg^{-1} (i.p.), 2 h apart[9]					None (all)	Expression (↑)		None (all)	
4 x 40.0 mg kg^{-1} (i.p.), 2 h apart[10]					None (all)	None (all)		None (all)	
4 x 25.0 mg kg^{-1} (s.c.), 2 h apart [11]	Mouse, Swiss CD-1	26.0 (±2.0)	FCX, STR	+3 days		Binding (FCX) (↓)			
4 x 50.0 mg kg^{-1} (s.c.), 2 h apart [11]						Binding (all) (↓)			

Dosing, regimen	Species, strain	Room temp. (°C)	Brain area (s)	Time point of effect	Effect on…				
					DA	DAT	DOPAC or HVA	TH	DA metabolism
3 x 25.0 mg kg^{-1} (s.c.), 2 h apart [11]	Mouse, Swiss CD-1	26.0 (±2.0)	FCX, STR	+3 days		Binding (all) (↓)			
4 x 40.0 mg kg^{-1} (i.p.), 2 h apart [9]	Mouse, C57BL/6	Not provided	STR	+7 days	None (all)	Expression (↑)		None (all)	
4 x 25.0 mg kg^{-1} (i.p.), 2 h apart [12]	Mouse, Swiss CD-1	26.0 (±2.0)	FCX, STR			(FCX) (↓)		(FCX) (↓)	
4 x 25.0 mg kg^{-1} (s.c.), 2 h apart [11]						None (all)			
4 x 50.0 mg kg^{-1} (s.c.), 2 h apart [11]						Binding (all) (↓)			
3 x 25.0 mg kg^{-1} (s.c.), 2 h apart [11]						Binding (FCX) (↓)			
2 x 30.0 mg kg^{-1} (i.p.), 4 consecutive days [7]	Mouse, C57BL/6	20.0 – 23.0	FCX, HIP, STR	+14 days	None (all)		HVA (STR) (↓)		

1.2.2.6 In vitro neurotoxicity of mephedrone

The cytotoxic profile of mephedrone has also been elucidated in part via *in vitro* assays, in which it has been shown to decrease cell viability and increase lactate dehydrogenase (LDH) release in SH-SY5Y cells (den Hollander *et al.*, 2014b), indicative of cellular death. Likewise, mephedrone demonstrably increases cell membrane toxicity at a greater concentration (IC_{50} > 2.0 mM), and depletion of adenosine triphosphate (ATP) at a lesser concentration (IC_{50} = 0.53 mM), than MDMA (IC_{50} = 0.74 and 0.88 mM, respectively), in C2C12 cells (Zhou *et al.*, 2019). Similarly, a mitochondrial 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) reduction assay in SH-SY5Y cells revealed mephedrone exhibited a median lethal dose (LC_{50} = 2.87 mM), ranking between amphetamine (LC_{50} = 3.23 mM) and methamphetamine (LC_{50} = 2.48 mM) (Soares *et al.*, 2019).

Other measures which have been applied to the study of mephedrone's cytotoxic profile *in vitro* include markers of mitochondrial function (formazan production from MTT) and free radical formation (reduced form glutathione), as have been employed in the study of the structurally-related cathinone phthalimide (Lantz *et al.*, 2017), whilst Wistar rat hepatocytes have been utilised as a medium to study the effects of 3-methylmethcathinone (3-MMC) (Dias da Silva *et al.*, 2019). Consistent with the *in vivo* observations of serotonergic deficits and impairments of neurogenesis in hippocampus (Ciudad-Roberts *et al.*, 2016b), inhibition of cell growth has been noted in mouse HT-22 hippocampal cells exposed to mephedrone's structural analogue 3-fluoromethcathinone (3-FMC) (Siedlecka-Kroplewska *et al.*, 2014).

1.2.2.7 Effect of other drugs on MDMAs neurotoxicity

Quantification of 5-HT content and SERT density, via HPLC and [^{3}H]-paroxetine binding, respectively, revealed an efficacy of the phosphodiesterase 5 inhibitor (PDE5) sildenafil (Viagra®) (1.5, 8.0 mg kg^{-1}, p.o.) to prevent MDMA's (3 x 5.0 mg kg^{-1}, two

hours apart) induction of neurotoxicity on serotonergic neurons in striatum, hippocampus and frontal cortex of male Wistar rats when administered 30 min prior to MDMA (Puerta *et al.*, 2009); and at the larger dose (8.0 mg kg^{-1}), 24 hrs prior to MDMA (Puerta *et al.*, 2012). In both cases, pre-administration of sildenafil produced no augmentation of MDMA-induced hyperthermia, the magnitude of which is typically related to the extent of long-term damage (Goni-Allo *et al.*, 2008). Alternatively, the authors posited that sildenafil exerted its neuroprotective effect (from MDMA) via inhibiting hydrolysis of cyclic guanosine monophosphate (cGMP), consequentially activating protein kinase G (PKG) (Corbin & Francis, 1999), and inducing proteins both antiapoptotic and antioxidative (Abdul & Butterfield, 2007), epigenetically (Andoh *et al.*, 2003). This suggestion is compounded in that: inhibition of PKG with KT5823 (2.0 nmol, intrastriatal microinjection) prevented sildenafil's protective effect; and activation of PKG with 8-Br-cGMP (20.0 nmol, intrastriatal microinjection) replicated sildenafil's protective effect, without augmenting MDMA-induced hyperthermia. There is no preclinical data for sildenafil's augmentation of mephedrone's effects (Hunter *et al.*, 2014; Chan *et al.*, 2015; Thurtle *et al.*, 2016; Giorgetti *et al.*, 2017).

Similarly, the SSRI fluoxetine (10.0 mg kg^{-1}, i.p.) (Prozac®) was demonstrated to protect against the neurotoxic effect of a high dose of MDMA (40.0 mg kg^{-1}, s.c.) on 5-HT and 5-HIAA content of hippocampus, striatum, frontal and somatosensory cortices two weeks later, with no effect on core body temperature (Malberg *et al.*, 1996). When pre-administered to rats, fluoxetine exerted this neuroprotection via inhibition of CYP2D6 (Jeppesen *et al.*, 1996), thereby preventing MDMA's metabolism to MDA.

1.2.2.8 Effect of other drugs on mephedrones neurotoxicity

In mice, co-administration of mephedrone (2.5 mg kg^{-1}) and nicotine (0.05 mg kg^{-1}) has been suggested to increase lipid peroxidation in hippocampus and prefrontal cortex, as indicated by pro-oxidative effects on these regions which include increases in malondialdehyde and decreased catalase activity (Budzynska *et al.*, 2015). Similarly,

mephedrone-induced increases in hippocampal lipid peroxidation, subsequent serotonergic and dopaminergic depletions in hippocampus and frontal cortex (one week), and impairment of neurogenesis (four weeks) were all potentiated upon co-administration of ethanol (Ciudad-Roberts *et al.*, 2016b). However, ethanol had no effect on the antioxidant response in frontal cortex. Combined, these data suggest an inefficiency in the antioxidant response upon nicotine or ethanol's co-administration, and the potential for these drug combinations to potentiate mephedrone-induced oxidative damage.

Elsewhere, mephedrone potentiated depletions of striatal DA, DAT and TH content induced by amphetamine, methamphetamine and MDMA (Angoa-Perez *et al.*, 2013), whilst none of these substances caused serotonergic depletions (with the exception of methamphetamine and MDMA on 5-HIAA and 5-HT, respectively) or increased microglial activation of the hippocampus (Angoa-Perez *et al.*, 2014).

Collectively, these data illustrate the importance of examining the long-term effects of mephedrone on brain and non-brain markers of toxicity, and whether the co-administration of mephedrone with other compounds serves to enhance or ameliorate these effects. Indeed, the capacity of mephedrone's effects to potentiate and be potentiated by other compounds is of great concern given the known propensity of its consumption alongside amphetamines, as well as licit compounds such as ethanol, nicotine and caffeine (interaction with the latter will be examined in the current thesis). In relation to the brain, the effects should be determined using measures which include markers of oxidative stress, monoaminergic content and neurogenesis.

1.3 Metabolism & Pharmacokinetics

1.3.1 Effect of mephedrone on metabolic activity

The effect of a drug on metabolic activity can be discerned through metabolomic profiling. In humans, nasal administration of mephedrone (100.0 mg) in a controlled experimental setting precipitated an increase in long-chain fatty acids, and a decrease in bile acids, detected via plasma sampling (Steuer *et al.*, 2020). These effects were more akin to those following amphetamine than MDMA (orally administered at doses of 40.0 and 125.0 mg, respectively), and potentially indicate the degradation of storage fats such as triglycerides as a consequence of mephedrone's metabolic energy demands, and consequently an increased demand for the use of bile acids to transport these free storage fatty acids. Mephedrone has recently been incorporated into a machine-learning algorithm developed to accurately predict and quantify the effects of newly-emerging psychoactive substances (Olesti *et al.*, 2019).

1.3.2 Metabolism of mephedrone

Analyses of human liver cells (Pedersen *et al.*, 2013) and rat hepatocytes (Khreit *et al.*, 2013) *in vitro*, as well as blood plasma and urine of Wistar (Meyer *et al.*, 2010) and Sprague-Dawley rats (Martinez-Clemente *et al.*, 2013) indicate mephedrone is metabolised to form up to 17 metabolites **(Fig. 1.10)**, with several other metabolites having been detected exclusively in human urine (Pozo Ó *et al.*, 2015). Difficulty in detecting mephedrone at sufficient levels in urine following administration (50.0 – 200.0 mg, oral or intranasal) has led Olesti *et al.* to suggest using the major metabolite 4'-carboxy-mephedrone as a complementary marker for the presence of mephedrone (Olesti *et al.*, 2020).

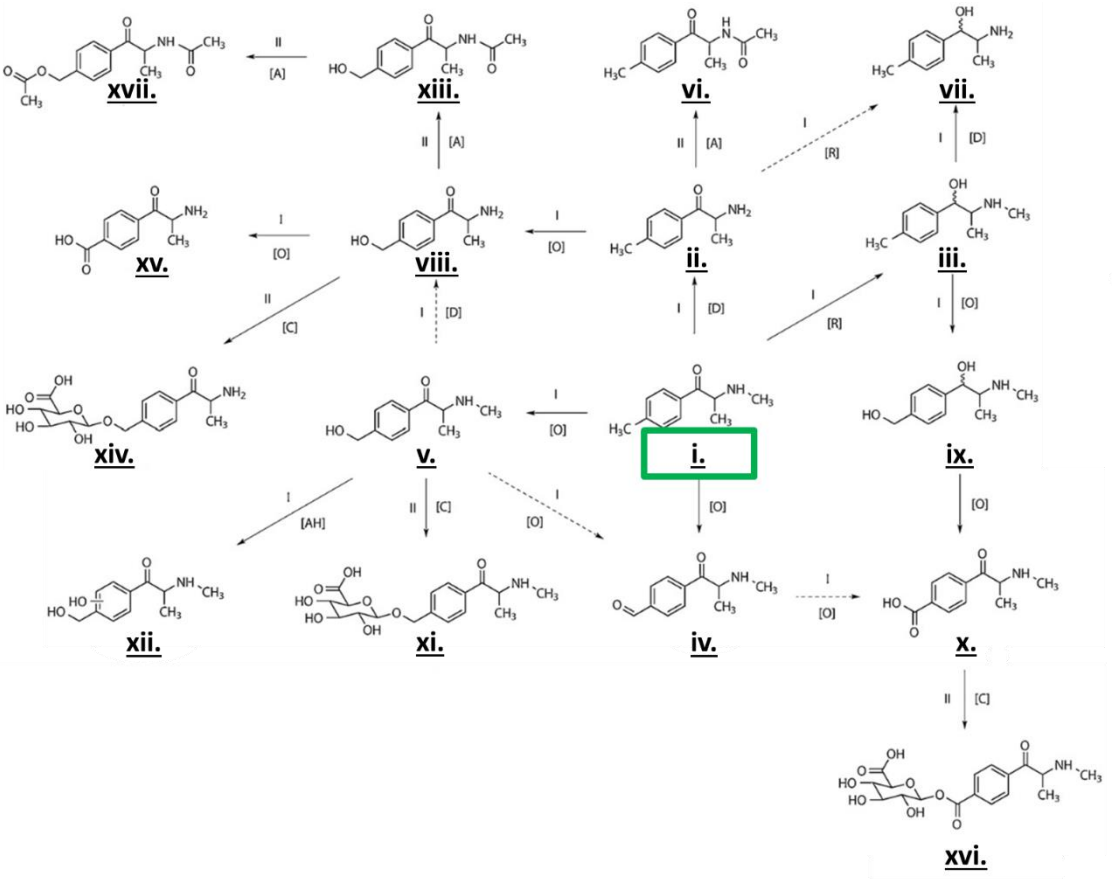


Figure 1.10 The potential routes of Phase I and II metabolism of mephedrone in Sprague-Dawley rat liver hepatocytes. In total, 17 metabolites, including mephedrone (**i.**), were detected: nor-mephedrone (**ii.**); 4'-methylephedrine (**iii.**); unspecified metabolite produced via oxidation (**iv.**); 4'-(hydroxymethyl)methcathinone) (**v.**); minor acetyl metabolite (**vi.**); unspecified phase I metabolite produced via reduction (**vii.**); 4'(hydroxymethyl)cathinone (**viii.**); unspecified phase I metabolite produced via oxidation (**ix.**); 4'-(carboxy)methcathinone (**x.**); unspecified phase II glucurodinated metabolite produced via conjugation (**xi.**); unspecified phase I metabolite produced via aromatic hydroxylation (**xii.**); minor acetyl metabolite (**xiii.**); glucuronide of 4'-(hydroxymethyl)cathinone (**xiv.**); unspecified phase I metabolite produced via oxidation (**xv.**); unspecified phase II acylglucuronide metabolite produced via conjugation (**xvi.**); minor acetyl metabolite (**xvii.**). Reactions involved in the metabolism of mephedrone include: acetylation [A]; reduction [R]; demethylation [D]; oxidation [O]; conjugation [C]; and aromatic hydroxylation [AH]. Figure adapted from Khreit *et al.* (2013).

Like MDMA (Tucker *et al.*, 1994), the cardinal enzyme implicated in mephedrone's degradation is cytochrome P450 2D6 (CYP2D6) (Pedersen *et al.*, 2013; Linhart *et al.*, 2016). Typically administered orally at a dose of 100.0 – 200.0 mg (similar to a recreational dose of MDMA; 140.0 – 180.0 mg, 1 – 2 tablets) and eliciting peak effects within 30 to 45 min (Papaseit *et al.*, 2016), recreational use of mephedrone differs in that it is more rapidly re-administered by users (Schifano *et al.*, 2011), such as 49.5% of a surveyed group of UK-based users who alleged to have consumed between 0.5 – 1.0 g during a single session (lasting between three to 12 hours in 75.6 % of cases) (Winstock *et al.*, 2011b). This re-dosing is attributable to the short-lasting psychoactive augmentation by mephedrone, which is rapidly taken up into and cleared from rat brain (Aarde *et al.*, 2013), and is in agreement with measurements of peak plasma (Martinez-Clemente *et al.*, 2013; Miller *et al.*, 2013) and brain (Aarde *et al.*, 2013) concentrations of the drug within one hour in rats.

Importantly, detection of both mephedrone and nor-mephedrone in rat blood serum, brain, lungs, and liver for up to four and seven to eight hours post-administration, respectively, indicate the potential of additive effects of subsequent doses at these concentrations, thus rendering them a matter of interest for toxicological study of these tissues (Sichova *et al.*, 2017). More recent data from humans has also detected mephedrone and 4-OH-mephedrone at reportedly high levels in blood plasma three hours following nasal administration of mephedrone (100.0 mg) (whilst still being detectable at six hours) (Steuer *et al.*, 2020), and at low but detectable levels (12.75 ng ml^{-1}) ten hours following oral administration (200.0 mg) (Papaseit *et al.*, 2019). Further, analysis with LC-MS/MS has detected mephedrone in fingerprints up to three days following administration (Czerwinska *et al.*, 2020).

Similarity in plasma concentrations (approx. 2000.0 ng mL^{-1}, equivalent to 0.011 mM) presented following toxic doses of mephedrone (Maskell *et al.*, 2011; Schifano *et al.*, 2012) and MDMA (Henry *et al.*, 1992) in humans further indicate the relevance of comparative study between these two drugs, though a number of important

differences must be appreciated. Firstly, unlike MDMA, which is more rapidly metabolised in rats than in humans (Baumann *et al.*, 2009), mephedrone appears to be degraded at a similar rate in both. Secondly, consequential of its lipophilic profile, mephedrone exhibits approximately two-fold greater permeability of the blood-brain barrier (BBB) (Simmler *et al.*, 2013), and is thus more readily taken up into, and subsequently cleared from the brain (Aarde *et al.*, 2013), complementing the more rapid onset and decline of psychoactive effects reported by users, and thus the accompanying propensity toward re-dosing (Schifano *et al.*, 2012). Thirdly, MDMA's propensity to induce neurotoxicity is generally agreed to be incurred as a consequence of its metabolism; specifically, via oxidation: wherein the catechol metabolites of MDMA lose electrons to generate quinone metabolites, which increase production of free radical (reactive oxygen (ROS) and reactive nitrogen species (RNS)). Although a recent study found mephedrone to elicit increased ROS formation in rat cortex, hippocampus and cerebellum, accompanied by mitochondrial dysfunction (Naserzadeh *et al.*, 2019), preclinical literature examining this effect of mephedrone is currently lacking. Lastly, whereas data concerning nor-mephedrone's pharmacological effects is scarce, MDA – primary metabolite of MDMA – is shown to exhibit a rather similar pharmacological profile to MDMA itself, and is an entactogen is its own right. Meanwhile, a recent study of C57BL/6J mice calculated the LD_{50} of mephedrone to be higher (118.8 mg kg^{-1}) than that of both methamphetamine (84.5 mg kg^{-1}) and MDMA (100.9 mg kg^{-1}) (Muskiewicz *et al.*, 2020), though this study employed an acute dosing paradigm which fails to account for the human practice of re-dosing.

1.3.3 Acute effect of mephedrone on gut microbiome

In relation to the gut microbiome, the effect of mephedrone (total of 40.0 mg kg^{-1}, i.p., across four injections two hours apart) on DNA from caecum contents has been examined via 16S rRNA sequencing. A significant decrease is noted in α-biodiversity (defined as the mean diversity of species in differing habitats or sites, within a local scale: in the present case, the human gut) one day following drug administration,

which returned to control levels by two days, as well as moderate increases in the diversity of some gut microbiome (*Barnesiella* and *Bacteroidales*) seven days following treatment (Angoa-Pérez *et al.*, 2020).

1.3.4 Toxicity in peripheral organs

Whilst data concerning mephedrone's propensity for neurotoxicity are lacking, investigation of its effect on HepG2 cells (an immortalised cell line comprising human liver cancer cells) have found mephedrone-induced reductions in cell count (Richter *et al.*, 2019) and increased mitochondrial dysfunction (Luethi *et al.*, 2017), with these effects observed in response to concentrations of 2.5 and 2.0 mM, respectively. Similarly, adolescent exposure to mephedrone (10.0 mg kg^{-1}, i.p.) has been shown to affectdegenerative changes in both liver and kidney tissue of Wistar rats (Joanna *et al.*, 2017), which were exacerbated by subsequent administration of morphine in adulthood, indicating lasting damage to peripheral organs as a consequence of adolescent administration. In Swiss mice, an acute dose of mephedrone (2.5 – 10.0 mg kg^{-1}, i.p.) induced pro-oxidative effects in the liver, kidneys, heart and spleen, one hour post-injection, characterised by a reduction in ascorbic acid and total antioxidant capacity (Tarkowski *et al.*, 2018). A cardiotoxic effect has also been suggested, with findings of mitochondrial damage and ROS production in heart cardiomyocytes incubated with mephedrone (Naserzadeh *et al.*, 2018). These data collectively indicate mephedrone's capacity to produce potentially long-lasting cytotoxicity in the peripheral organs, which is of concern for human users.

1.4 Physiological, Behavioural and Cognitive effects

1.4.1 Locomotor activity (LMA)

Consistent with its action to increase dopamine efflux of NAcc, mephedrone has been shown to effect increases in locomotor activity in rodents (Wright *et al.*, 2012; Shortall

et al., 2013b; Green *et al.*, 2014; Pail *et al.*, 2015; Nguyen *et al.*, 2016; Shortall *et al.*, 2016a; Javadi-Paydar *et al.*, 2018; Benturquia *et al.*, 2019), which appeared to be particularly dependent on 5-HT mechanisms (Lopez-Arnau *et al.*, 2012; Shortall *et al.*, 2016b). The length of this hyperlocomotion was relatively shorter than that elicited by MDMA, consequential of mephedrone's shorter plasma half-life and metabolic rate (Green *et al.*, 2014), whilst of lesser magnitude than that affected by amphetamine, which effects comparatively greater dopamine release (Kehr *et al.*, 2011). Typically, increases are noted within 15 minutes of administration, returning to baseline within one hour.

Caffeine, which is also evidenced to enhance dopamine efflux of NAcc indirectly (Solinas *et al.*, 2002), has been shown to potentiate mephedrone-induced hyperlocomotion in rats (Shortall *et al.*, 2016a).

1.4.1.1 Locomotor sensitisation

Repeated administration of psychostimulants elicits a progressive increase in locomotor activity, relative to the initial administration, and is referred to as sensitisation of the locomotor response. Demonstration of this response following an interruption or washout period (typically of several days) is indicative of the abuse liability of the drug.

Administration of mephedrone at a high dose (30.0 mg kg^{-1}) failed to induce locomotor sensitisation in Wistar rats exposed across four (twice daily, six hours apart) (den Hollander *et al.*, 2014a) or ten day (daily dose) regimens (Motbey *et al.*, 2012a), though this is to be anticipated at the doses employed (den Hollander *et al.*, 2014a; Green *et al.*, 2014). At lower doses, sensitisation was manifest in Sprague-Dawley rats as an enhancement of: repetitive movements following daily doses across five-day-constant (15.0 mg kg^{-1}, i.p.) and seven-day-variable protocols (i.e. 15.0 mg kg^{-1} on days one and

seven, and 30.0 mg kg^{-1} on days two through six, i.p.) (Gregg *et al.*, 2013a); horizontal activity following seven consecutive daily sub-threshold doses (0.5, 1.0, 2.0 mg kg^{-1}, i.p.) (Berquist *et al.*, 2016); and ambulatory activity following five consecutive daily sub-threshold doses (0.5 mg kg^{-1}, i.p.) and a ten day abstinence period (Lisek *et al.*, 2012). Sensitisation of horizontal activity was also observed in female CD-1 mice following seven daily (excluding day two) injections (3.0 mg kg^{-1}, s.c.) (Berquist *et al.*, 2015), and in male Swiss mice administered mephedrone (1.0 mg kg^{-1}, i.p.) in an intermittent nine day protocol (i.e. days one, three, five, seven and nine). Further, locomotor activity was enhanced in Lister-Hooded rats administered mephedrone (10.0 mg kg^{-1}, i.p.) twice weekly, on consecutive days, across a three week period; a dosing regimen reflective of human recreational use (Shortall *et al.*, 2013b). However, despite its efficacy to produce conditioned-place-preference (CPP) at the same doses (1.0, 10.0 µM) in *Dugesia tigrina*, sensitisation to mephedrone was not observed two, six, or 13 days following withdrawal from a ten day alternate-day dosing protocol (Hutchinson *et al.*, 2015), though such a difference might be attributable to dosage (perhaps too high) or species differences.

Following a ten day abstinence period, enhancement of the locomotor response was also observed following cocaine challenge (5.0, 15.0 mg kg^{-1}, i.p.) in Sprague-Dawley rats previously dosed daily and consecutively with mephedrone across seven (2.0 mg kg^{-1}, i.p.) (Berquist *et al.*, 2016) and five days (15.0 mg/kg, i.p.) (Gregg *et al.*, 2013b), respectively. Interestingly, at the same doses, Gregg *et al.* observed no such enhanced response following mephedrone challenge of animals pre-treated with cocaine, or of mephedrone pre-treated animals challenged with methamphetamine (15.0 mg kg^{-1}, i.p.), perhaps indicating substance specificity of mephedrone's profile to produce cross-sensitisation. Supporting this, mephedrone (1.0 or 5.0 mg kg^{-1}, i.p.), administered over seven consecutive days, did not elicit sensitisation of the locomotor response to a challenge dose (1.0 or 5.0 mg kg^{-1}, i.p.) following ten days of abstinence. However, when co-administered with MDMA (3.0 mg kg^{-1}, i.p.) throughout this period, mephedrone potentiated the locomotor response to a challenge dose of MDMA on day 17, relative to rats treated solely with MDMA (Bullock *et al.*, 2019).

However, earlier studies found horizontal activity to be significantly enhanced in mephedrone-pre-treated (3.0 mg kg^{-1}, s.c.) female CD-1 mice following D-amphetamine challenge (1.0 mg kg^{-1}, s.c.) (Berquist *et al.*, 2015), and in nicotine-pre-treated (0.5 mg kg^{-1}, s.c.) male Swiss mice, following mephedrone challenge (1.0 mg kg^{-1}, i.p.) (Budzynska *et al.*, 2015) (protocols aforementioned). The co-administration of mephedrone with MDPV and methylone (3.3 mg kg^{-1} each, i.p.) every second day over a 14 day period produced locomotor sensitisation on day 14, characterised by decreased activity, in rats (Allen *et al.*, 2019), indicating the development of locomotor sensitisation to the combination of these compounds (i.e. sensitisation to the "bath salt" mixture).

Several possibilities, as indicated initially by the discrepancies in dosage sizes, might underlie the inconsistencies in the literature of sensitisation and cross-sensitisation. Such possibilities include the variability of dosing protocols and regimens, and routes of administration, although this latter factor is unlikely to have had an effect. The preclinical study of the phenomena of sensitisation (as well as cross-sensitisation) of mephedrone – both to and from other compounds – is of particular importance considering the real-world behaviour of recreational mephedrone users, much of whom are polydrug users, who likely in many cases have: converted to using mephedrone, for instance under the false pretence of it being safer in comparison to other conventional illicit stimulants; or changed from using mephedrone-based products exclusively to using more established drugs, and/or combining mephedrone with other stimulants.

1.4.1.2 Stereotyped behaviour

The serotonin syndrome is an adverse effect which manifests as a consequence of excess serotonin release in the central and peripheral nervous system. In humans, this syndrome is characterised by agitation, tachycardia, diaphoresis, fever, hypertension

and clonus, amongst other symptoms (Boyer & Shannon, 2005) **(Fig. 1.11)**, and typically results from the co-administration of two or more drugs which precipitate the increase of synaptic 5-HT content via different mechanisms. Fatalities consequential of this syndrome have been observed as a result of MDMA abuse (Henry *et al.*, 1992; Coore, 1996) and following the co-consumption of antidepressants with other drugs such as benzodiazepines, opioids, and caffeine (Hernandez *et al.*, 1995; Power *et al.*, 1995).

As mephedrone increases synaptic 5-HT levels via its action as a substrate and blocker at SERT, it is possible that this syndrome can result from its consumption, with the likelihood of manifestation being enhanced upon co-consumption of other serotonergic drugs (Bartlett, 2017). Indeed, such observations have been made in the case of a 41 year old woman using a bath salts composition (Mugele *et al.*, 2012), a 24 year old woman consuming a capsule containing methylone and butylone sold as "Ecstasy" (Warrick *et al.*, 2012) and of a mephedrone user with a medical prescription for fluoxetine (Garrett & Sweeney, 2010).

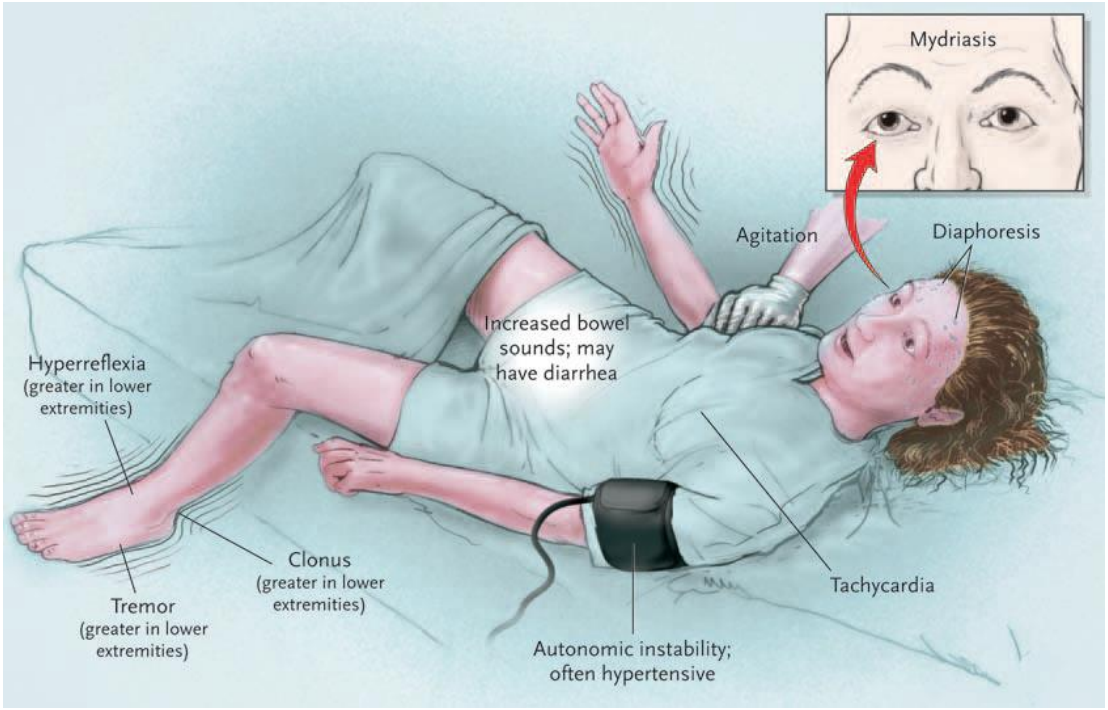


Figure 1.11 Illustration of a patient presenting with moderate to severe serotonin syndrome. The syndrome may be characterised by symptoms such as agitation, tachycardia, hypertension, shivering, diaphoresis, mydriasis, as well as hyperkinetic neuromuscular activity, ranging from intermittent tremoring and myoclonus to hyperreflexia. Image taken from Boyer & Shannon (2005).

In rats, the serotonin syndrome is characterised by hyperactivity, piloerection, salivation, protrusion of the eye, defecation, penile erection and ejaculation, as well as numerous stereotyped behaviours including head weaving and reciprocal forepaw treading (Jacobs & Klemfuss, 1975; Goodwin & Green, 1985; Haberzettl *et al.*, 2013) **(Fig. 1.12)**, both of which are dose-dependently elicited by MDMA (Callaway *et al.*, 1990; Fone *et al.*, 2002; Bull *et al.*, 2004) and correlate with MDMA-induced dialysate 5-HT content in striatum (Baumann *et al.*, 2008b) – an area strongly implicated in motor activity (Schultz, 2000). Consistent with its pharmacological profile, and anecdotal evidence in human users, acute administration of mephedrone (10.0 mg kg^{-1}) has been found to elicit reciprocal forepaw treading in rats (Baumann *et al.*, 2012).

Literature indicates these stereotyped behaviours can be prevented via blockade of the $5\text{-}HT_{1A}$ receptor. For instance, pre-administration of WAY-100,635 has been shown to prevent 8-OH-DPAT-induced reciprocal forepaw treading (Forster *et al.*, 1995; Bardin *et al.*, 2001; Kawano *et al.*, 2015) and lateral head weaving (Sato *et al.*, 2012), whilst the latter behaviour has also been prevented by the antagonist and partial agonists pindolol (Yamaguchi *et al.*, 1987) and methysergide (Fujii *et al.*, 1991), respectively.

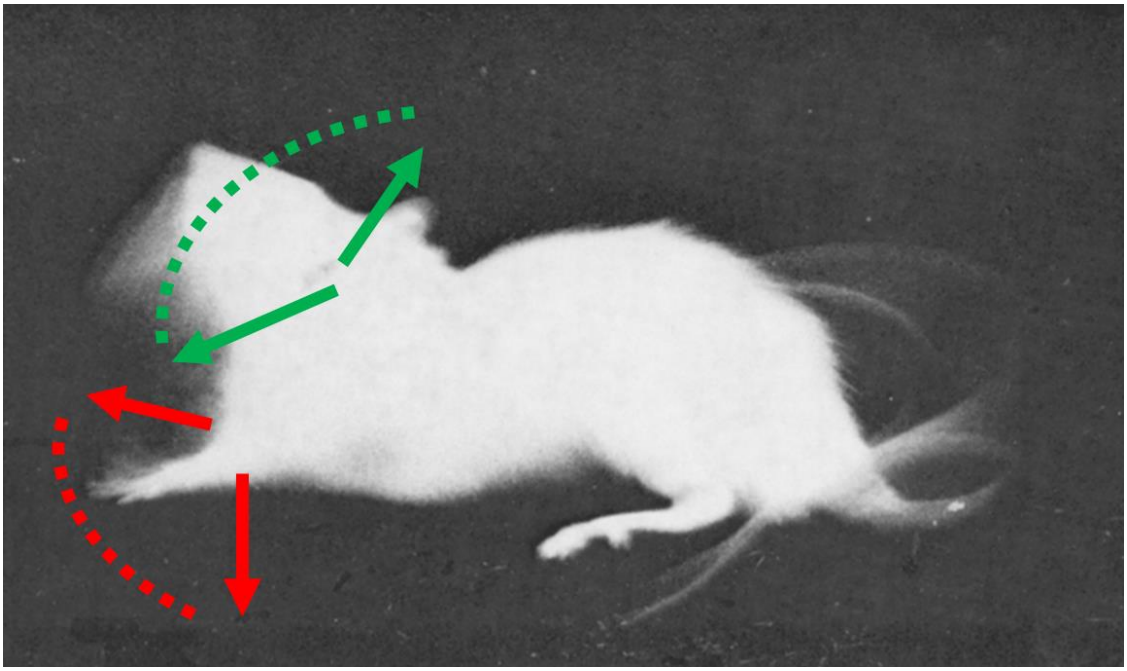


Figure 1.12 Time-lapse image of rat exhibiting serotonin syndrome following administration of pargyline (50.0 mg kg^{-1}, i.p.), followed one hour later by L-tryptophan (150.0 mg kg^{-1}, i.p.), annotated to illustrate stereotyped behaviours of interest to the present thesis. Symptoms exhibited include tremor (body is blurred), lateral head weaving (highlighted in green; also vertical in this example), reciprocal forepaw treading (highlighted in red; lateral movement of anterior limbs), Straub tail (in this example the tail is lashing, whilst in others it may be erect) and hindlimb abduction (does not move, and therefore not blurred, as in case of forelimbs). Image adapted from Jacobs & Klemfuss (1975).

Complementing the observation that MDMA's induction of these stereotyped behaviours correlates with striatal 5-HT content (Baumann *et al.*, 2008b), reduction in PCP-induced lateral head weaving has been noted following lesioning of striatum by bilateral electrocoagulation (Nabeshima *et al.*, 1983a), and administration of 5,6-dihydroxytryptamine (5,6-DHT) (Nabeshima *et al.*, 1983b), whilst striatal transection prevented lateral head weaving following pargyline with L-tryptophan (Jacobs & Klemfuss, 1975). Interestingly, striatal lesioning with the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) has also been shown to reduce lateral head weaving elicited by amphetamine (Andrews *et al.*, 1982) and PCP (Nabeshima *et al.*, 1983b),

respectively, suggesting a role for dopaminergic systems in the serotonin syndrome also.

Given the low affinity for mephedrone at the 5-HT_{1A} receptor (Eshleman *et al.*, 2013; Simmler *et al.*, 2013), it is more likely that synaptic 5-HT, increased by mephedrone, binds to postsynaptic 5-HT_{1A} receptors and elicits these stereotyped behaviours. Concerningly, the co-consumption of caffeine might enhance this pharmacological effect. As an antagonist at adenosine A_1 and A_2 receptors, caffeine increases extracellular levels of 5-HT indirectly both *in vitro* and *in vivo* (Nehlig *et al.*, 1992), and demonstrably enhances MDMA-induced 5-HT efflux (Gorska & Golembiowska, 2015). As such, the co-consumption of caffeine with mephedrone, as occurs amongst recreational human users, constitutes a health concern for the occurrence of the serotonin syndrome. It is important to note that the stereotyped behaviours observed in these preclinical studies are distinct from other stereotyped behaviours which are mediated via 5-HT_{2A} receptors, which include head twitches, wet dog shakes and back muscle contractions (Fone *et al.*, 1991; Ninan & Kulkarni, 1998; Rojas-Corrales *et al.*, 2007; Umeda *et al.*, 2007). Although mephedrone exhibits binding affinity in the micromolar range at the 5-HT_{2A} receptor, there is currently no preclinical data characterising the efficacy of mephedrone to activate this receptor to achieve these effects.

1.4.2 Body temperature

Hyperthermia is a commonly reported, and likely adverse consequence of human MDMA consumption (Parrott, 2012). Pre-clinical data from the rat indicates augmentation of body temperature following MDMA is mediated by dopaminergic and noradrenergic systems, and is contingent on a variety of factors, whereby hyperthermia – likely mediated via downstream activation of endogenous neurotransmitters on D_2 receptors, α_1-, and β-adrenoceptors – follows from administration at a high or repeated dose, as well as within a high-ambient room

temperature (> 25.0 °C) or in group-housed animals. Conversely, hypothermia – likely mediated via D_1 receptors and α_2-adrenoceptors – follows from administration of MDMA at a low or acute dose, or at a cold (Dafters, 1994) or low-ambient room temperature, and in animals housed individually (review (Green *et al.*, 2005)). Interestingly in the case of repeated administration, a biphasic profile has been displayed in animals treated at a low-ambient room temperature, whereby induction of an initial hypothermic reaction gives way to neutralisation and hyperthermia at subsequent doses (Rodsiri *et al.*, 2011). In mice, the initial hypothermic response to mephedrone (4 x 40.0 mg kg^{-1}, i.p., two hours apart) was prevented by depletion of brain 5-HT content via genetic knockout of the TPH2 gene (Anneken *et al.*, 2019), though the later hyperthermic response remained present in these animals, indicating a serotonergic component to the hypothermic response to mephedrone in mice.

Despite experiencing unpleasant changes in temperature (Dargan *et al.*, 2011; Vardakou *et al.*, 2011) – including hot flushes, sweating (Schifano *et al.*, 2011), blue lips and fingers (Ahmed *et al.*, 2010), cold peripheries and skin discolouration (Dick & Torrance, 2010) – recreational users of mephedrone, unlike those of MDMA, seldom report a hyperthermic reaction to the drug (Dargan *et al.*, 2011; Wood *et al.*, 2011).

Preclinical data indicate mephedrone's augmentation of core body temperature is also contingent on dosing regimen and context. Administration at a low ambient room temperature (Miller *et al.*, 2013; Shortall *et al.*, 2013a), as well as at acute and/or low doses (Aarde *et al.*, 2013; Shortall *et al.*, 2013a; Lopez-Arnau *et al.*, 2015; Shortall *et al.*, 2016b; Muskiewicz *et al.*, 2020) consistently renders a hypothermic response, which in rats appears to be mediated via serotonergic neurons and downstream 5-HT_{1A} agonism, as indicated by effective prevention of this response by the 5-HT neurotoxin 5,7-dihydroxytryptamine and 5-HT_{1A} antagonist WAY-100,635 (Shortall *et al.*, 2016b).

This hypothermic reaction is abolished upon administration of subsequent doses of mephedrone (Shortall *et al.*, 2016b), and is often converted into hyperthermia (Hadlock *et al.*, 2011; Angoa-Perez *et al.*, 2012; Baumann *et al.*, 2012; Martinez-Clemente *et al.*, 2014; Anneken *et al.*, 2017a), which also occurs following administration at high doses (den Hollander *et al.*, 2014a; Grecco & Sprague, 2016; Zona *et al.*, 2016), and at normal (Baumann *et al.*, 2012) to high ambient room temperature (Hadlock *et al.*, 2011). This hyperthermia is evidenced to be effected via downstream activation of α_1- and β-adrenoceptors (Zona *et al.*, 2016). The co-administration of caffeine also serves to convert mephedrone-induced hypothermia to hyperthermia in Lister-Hooded rats (Shortall *et al.*, 2016a).

1.4.3 Self-administration/stimulation

The reinforcing properties and abuse liability of a drug can be indicated by the extent to which it is self-administered in rodent models. A behaviour learned via operant conditioning, self-administration of mephedrone (0.5 mg kg^{-1} per infusion) has been acquired in male (Vandewater *et al.*, 2015) and female rats (Creehan *et al.*, 2015; Marusich *et al.*, 2019). Importantly, mephedrone appears to exhibit more reinforcing properties than both MDMA and methylone in this paradigm (Creehan *et al.*, 2015; Vandewater *et al.*, 2015), as indicated by a greater number of self-infusions of the drug and presses of the drug-associated lever, suggesting a greater abuse liability. Likewise, at a dose identical to that of methamphetamine (0.24 mg kg^{-1} per infusion), mephedrone elicited self-administration in Sprague-Dawley rats exposed across eight days (four hours per day) to a lever-press paradigm, wherein discrimination of the rewarding lever was notably enhanced on day eight (10.71:1) relative to day one (2.65:1) (Hadlock *et al.*, 2011).

Intracranial self-stimulation (ICSS), also contingent on operant conditioning, entails the self-delivery of brief electrical impulses to the brain. In this methodology, the rewarding properties of a drug are indicated by its ability to lower the ICSS (or reward)

threshold, whilst increasing ICSS thresholds are indicative of a state of anhedonia, such as in the event of drug withdrawal. When delivered via i.p. injection (1.0 mg kg^{-1}) or vaporised e-cigarette format (200.0 mg ml^{-1}, in propylene glycol) to Wistar rats, mephedrone has been shown to lower the reward threshold (Nguyen *et al.*, 2016). Also, congruent with its binding affinity for DAT and SERT, mephedrone (10.0 mg kg^{-1}, i.p.) has been shown to exert both facilitation of low ICSS rates, and dose-dependent depression of high ICSS rates in Sprague-Dawley rats (Bonano *et al.*, 2014; Gregg *et al.*, 2015) and C57BL/6J mice (Robinson *et al.*, 2012), indicative of dopamine-mediated abuse-potential and 5-HT-mediated abuse-limitation, respectively. The relatively weak efficacy of mephedrone, compared to methcathinone, MDPV and methylone, to facilitate low ICSS rates was interpreted by Bonano *et al.* as an indication of mephedrone having a lower potential for abuse, though repeated exposure over a period of 31 days has been shown to coincide with an enhancement of ICSS facilitation, due to the development of tolerance (Suyama *et al.*, 2019). As such, these data collectively indicate that mephedrone exhibits reinforcing properties which may render it prone to abuse.

1.4.4 Drug discrimination

According to self-reports of users, mephedrone is qualitatively perceived as both an entactogen and stimulant, subjectively resemblant of MDMA, and to a lesser extent cocaine (Carhart-Harris *et al.*, 2011), respectively (Hadlock *et al.*, 2011), and in some cases exerts hallucinogenic effects (Winstock *et al.*, 2011b). The chemical structure of mephedrone, like methamphetamine, cathinone, MDMA and fenfluramine, is that of a substituted amphetamine. It is in respect of the common pharmacological bases of substances, and commonalities in effects reported by users in relation to them, that they be collectively employed in drug discrimination paradigms (Young, 2009).

1.4.4.1 Discrimination of mephedrone

Rats can be trained to discriminate mephedrone in Fixed Ratio (FR) schedules of reinforcement (Aarde *et al.*, 2013) (FR1-FR5); (Berquist *et al.*, 2017) (FR1-FR20); (Creehan *et al.*, 2015) (FR1); (DeLarge *et al.*, 2017) (FR20); (Varner *et al.*, 2013) (FR20)), with Wistar rats having been shown to acquire mephedrone as a discriminative stimulus more readily than they do MDMA (Creehan *et al.*, 2015). Following this acquisition, similarity of the subjective effects induced by other drugs are compared to those induced by mephedrone, and are assessed through the employment of alternative substances as substitution candidates.

Scant literature regarding the generalisation of mephedrone as a discriminative stimulus nonetheless serves to elucidate its pharmacology and qualitative effects **(Table 1.9)**. Collectively, these data indicate mephedrone is not substituted for by opioids (morphine and heroin) or N-methyl-D-aspartate (NMDA) antagonists (ketamine and PCP), but by prototypical stimulants (cocaine, methamphetamine, D-amphetamine), entactogens (MDMA and MDA), and other synthetic cathinones (MDPV), often in a dose-dependent manner (Varner *et al.*, 2013; Berquist *et al.*, 2017; DeLarge *et al.*, 2017; Erwin *et al.*, 2019; Saber *et al.*, 2019). Partial substitution for mephedrone has also been exhibited by the psychedelic hallucinogen LSD (Berquist *et al.*, 2017), with the generalisation in this instance perhaps attributable to a shared affinity of these compounds for the 5-HT_{2A} receptor; a suggestion compounded by the observation of mephedrone's dose-dependent substitution by MDA (DeLarge *et al.*, 2017), which also exhibits high affinity at the 5-HT_{2A} receptor. Substitution was not, however, observed for the psychedelic hallucinogen and 5-HT_{2A} agonist R-DOI (DeLarge *et al.*, 2017). Specifically, of the group of five animals tested with R-DOI, two were tested at the larger dose (1.0 mg kg^{-1}), which failed to effect significant increases in maximal mephedrone-lever responding (which was 52.9%). Extrapolation of inference from data of two subjects is objectively injudicious, and the shared pharmacological affinity of R-DOI necessitates further investigation of this substance

as a substitution candidate for mephedrone. Nonetheless, these findings have been replicated using DOI (Saber *et al.*, 2019).

The off-market appetite suppressant and substituted amphetamine, fenfluramine, which increases the synaptic presence of 5-HT via disruption of vesicular storage and reverse transport at SERT, partially and fully substituted for mephedrone, dependent on training dose employed (Varner *et al.*, 2013; Berquist *et al.*, 2017). This is likely attributable to the shared property of these two substances to elevate the synaptic content of 5-HT.

Table 1.9 Substitution capacities of drugs for mephedrone. Substitution capacities: full (significantly different from vehicle, > 80 %, dark grey); partial (significantly different from vehicle, < 80 %, light grey); none (denoted with /, white). Reference key: [1]DeLarge et al. (2017); [2]Berquist et al. (2017); [3]Varner et al. (2013); [4]Saber et al. (2019); and [5]Erwin et al. (2019).

Substitution drug	Mephedrone training dose (mg kg^{-1})											
	0.5		1.0		3.0		3.2					
Mephedrone	0.15 [4]	0.5 – 1.6 [4]	1.0 [2]		1.0 [2] 3.0 [2]		0.5 [4], 0.56 – 1.8 [1, 5]	1.6 – 5.0 [4] 3.2 [3] 3.2 – 10.0 [1, 5]				
(*S*)-mephedrone	0.05 – 0.15 [4]	0.5 [4]										
(*R*)-mephedrone	0.5 [4]	1.6 [4]										
Cocaine	5.0 [4]	10.0 – 15.0 [4]	3.0 [2]	10.0 [2]	10.0 [2] 20.0 [2]		1.8 – 10.0 [1] 5.6 – 18.0 [5] 10.0 [4]	10.0 [1] 10.0 – 32.0 [3]				
MDMA	0.5 [4]	1.6 – 9.0 [4]	1.0 [2]		1.0 [2]	3.0 [2]	1.8 [3]	3.2 [3] 5.0 – 9.0 [4]				
Methamphetamine	0.15 – 0.28 [4]	0.5 – 3.0 [4]	0.1 – 1.0 [2]	3.0 [2]	1.0 [2]		0.28 – 3.0 [4] 1.0 – 1.8 [3]					
Fenfluramine			3.0 [2]		3.0 [2]		1.0 – 3.2 [3]					
Morphine	/ [4]						/ [1, 5]					

PCP						/ [1, 5]	
MDPV			1.0 [2]	3.0 [2]	1.0 [2]	/ [1, 5]	
Amphetamine	0.03 – 0.5 [4]	1.0 – 2.0 [4]	1.0 [2]		1.0 [2]	/ [4]	1.8 – 3.2 [1]
LSD			/ [2]		0.1 – 0.2 [2]		
Ketamine						/ [1, 5]	
Heroin						/ [1, 5]	
DOI	0.1 – 1.0 [4]	2.0 [4]				/ [1]	2.0 [4]
Δ 9-THC						1.0 – 5.6 [1]	
MDA						1.8 [1]	3.2 [1]
Methylphenidate						3.2 – 10.0 [1]	
WAY 163909	2.0 [4]					/ [1]	
mCPP	0.3 – 1.6 [4]						
Amitriptyline						/ [5]	
Bupropion						18.0 – 32.0 [5]	

An apparent inconsistency observable in the data **(Table 1.9)** is the full substitution of a substance for mephedrone in animals trained to discriminate mephedrone at a lower dose, with this same dose (of the substitution candidate) failing to achieve any degree of substitution in animals trained to discriminate mephedrone at a higher dose. This is observable in data presented for D-amphetamine, methamphetamine and MDPV (and to a lesser extent for fenfluramine). At a dose of 3.0 mg kg^{-1}, for instance, MDPV fully substituted for mephedrone in animals trained to discriminate mephedrone at a low dose (1.0 mg kg^{-1}), whilst failing to achieve even partial substitution for mephedrone in animals trained to discriminate mephedrone at a higher dose (3.0 mg kg^{-1}). Further, at a dose which achieved partial substitution in both groups of mephedrone-trained animals (1.0 mg kg^{-1}) (Berquist *et al.*, 2017), MDPV did not significantly elevate mephedrone-lever responding in animals trained to discriminate mephedrone at a higher dose (3.2 mg kg^{-1}) (Varner *et al.*, 2013; DeLarge *et al.*, 2017).

In regards this apparent incongruence, it is pertinent to note that generalisation of a training substance to a variety of pharmacologically different substitution candidates is in a minority of cases more readily achieved following training at a lower dose of the

substance. For instance, capacity for generalisation of amphetamine has in some cases been found to be enhanced at lower doses, likely reflecting a relatively enhanced sensitivity in these animals to discriminate lower doses of subsequent substitution candidates (Stolerman *et al.*, 2011). Similarly, Saber *et al.* found that mephedrone was fully substituted for by d-amphetamine, MDMA, cocaine, as well as mephedrone in its racemic and enantiomeric forms, when rats were initially trained using a low dose (0.5 mg kg^{-1}), but was only substituted for by MDMA and mephedrone at a higher dose (3.2 mg kg^{-1}) (Saber *et al.*, 2019).

1.4.4.2 Mephedrone as a substitution candidate

In reverse, mephedrone has been shown to successfully function as a substitute candidate itself for cocaine in rats (Gatch *et al.*, 2013) and rhesus monkeys (Smith *et al.*, 2017), as well as for methamphetamine (Gatch *et al.*, 2013), d-amphetamine (Harvey *et al.*, 2017), MDMA, and a mixture of both (Harvey & Baker, 2016) in rats **(Table 1.10)**.

The capacity of mephedrone to substitute for, and be substituted for, numerous psychostimulants, including MDMA, further validate employment of MDMA data as a positive control in experiments, where applicable.

Table 1.10 Substitution capacity of mephedrone for other drugs. Substitution capacities: full (significantly different from vehicle, > 80 %, dark grey); none (denoted with /, white); data not divulged in cited publication (?); median effective dose (ED_{50}). Reference key: [1]Gannon & Fantegrossi (2016) (mouse, i.p.); [2]Gatch *et al.* (2013) (rat, i.p.); [3]Harvey & Baker (2016) (rat); [4]Harvey *et al.* (2017) (rat); [5]Smith *et al.* (2017) (rhesus monkey); and [6]Aarde *et al.* (2013) (rat).

Training drug	Training dose (mg kg^{-1})	Mephedrone dose (mg kg^{-1})	Mephedrone ED^{50}
Cocaine	**0.32** [5]	**/** [5]	**0.57** [5]
	10.0 [1, 2]	**3.0** [1]	**1.47** [1] **1.5** [2]
Methamphetamine	**0.1** [6]	**0.1** [6]	**?** [6]
	1.0 [6]	**?** [2]	**1.27** [2]
Amphetamine	**0.5** [4]	**2.0** [4]	**1.15** [4]
MDMA	**1.5** [4]	**2.0** [3]	**0.22** [3]
MDMA + amphetamine	**1.5 + 0.5** [3]	**2.0** [3]	**0.56** [3]

1.4.5 Conditioned-place-preference

Achieved via classical conditioning, CPP is indicative of the rewarding or motivational property of a substance (Prus *et al.*, 2009). Like other drugs of abuse, literature indicates a propensity for mephedrone to induce CPP in rats (Lisek *et al.*, 2012; Gregg *et al.*, 2015), mice (Karlsson *et al.*, 2014; Ciudad-Roberts *et al.*, 2015), planaria (Ramoz *et al.*, 2012; Vouga *et al.*, 2015) and crayfish (Gore *et al.*, 2020), and that racemic and (*R*)-enantiomeric mephedrone are more efficient in this function than is the (*S*)-enantiomer (Gregg *et al.*, 2015; Vouga *et al.*, 2015).

The rewarding value of numerous drugs, including methamphetamine (Higley *et al.*, 2011a; Higley *et al.*, 2011b), is demonstrably contingent on dopamine D_3 receptors. The expression of these receptors is evidenced to be contingent on brain-derived-

neurotrophic-factor (BDNF) (Guillin *et al.*, 2001), a protein whose presence is increased by psychostimulants (Graham *et al.*, 2007). Increased expression of the *Drd3* gene (which codes for the D_3 receptors) and BDNF mRNA has been shown to coincide with mephedrone-induced CPP, with prevention of these effects upon co-administration of D_3 (SB-277011A) and TrkB (ANA-12) receptor antagonists (Ciudad-Roberts *et al.*, 2015). In mice, mephedrone-associated memories have also been disrupted by infusion to the basolateral amygdala complex of Blebbistatin – a nonmuscle myosin II inhibitor (Briggs *et al.*, 2018).

In rats, the combination of sub-threshold doses of ethanol and MDMA has been shown to produce CPP (Jones *et al.*, 2010). Similarly in mice, low dose ethanol has been evidenced to potentiate CPP induced by high dose mephedrone (25.0 mg kg^{-1}, s.c.) (Ciudad-Roberts *et al.*, 2015); an effect which is not replicated with low dose mephedrone (5.0 mg/kg, i.p.) (Siivonen *et al.*, 2018). Caffeine – a compound also co-consumed in the recreational context – has been shown not to modify CPP score following cocaine (10.0 mg kg^{-1}, administration route not provided by author) administration to male C57BL/6 mice (Muniz *et al.*, 2017). These limited data provide an incomplete image of the ability of ethanol and caffeine to modify the rewarding and motivational properties of mephedrone in human users, and therefore remains an area of active interest, given the ubiquity of these compounds.

The exposure of rodents to psychostimulants during adolescence has been shown to potentiate CPP in adulthood. Administered for 28 days to adolescent male Sprague-Dawley rats, caffeine (throughout this time, rats were given access to a single bottle containing 0.3 g L^{-1} caffeine in tap water) precipitated a robust enhancement of the CPP score in response to a subthreshold dose of cocaine (7.5 mg kg^{-1}, i.p.) (O'Neill *et al.*, 2015), whilst mephedrone (10.0 mg kg^{-1}, i.p.) – administered once daily (PND 30-37) – rendered a potentiation in CPP induced by morphine (5.0 mg kg^{-1}) in adulthood (PND 60-64) (Joanna *et al.*, 2017). Given the popularity of recreational drug use among

adolescent and young adult users, it is important to understand the long-lasting effects of recreational drug use in these age groups, which may persist into adulthood.

1.4.6 Anxiety

Whilst variant degrees of agitation are a frequent symptom indicative of recent usage of a number of designer drugs (Weaver *et al.*, 2015), anxiety is often cited as an adverse effect of NPS (Sande, 2016) and synthetic cathinones (Prosser & Nelson, 2012; Assi *et al.*, 2017), and has been reported by mephedrone users as the most common of all adverse effects (Carhart-Harris *et al.*, 2011). Such is the frequency of this effect, that reports of anxiety are indicated to be significantly more prevalent in recreational users of mephedrone than of MDMA (Jones *et al.*, 2016). Additionally, mephedrone-induced anxiety is cited in the clinical presentations of a 25 year old man who utilised the drug intravenously for chemsex most weekends for four months (Dolengevich-Segal *et al.*, 2016), and a 22 year old man after having taken a product believed to comprise mephedrone the night prior (Lenz *et al.*, 2013). The capacity of mephedrone to elicit anxiety in these cases is of added concern given the increased incidence of anxiety in methamphetamine-using MSM (Schecke *et al.*, 2019), suggesting a potential additive effect upon co-consumption of these two compounds within a single chemsex session. Similarly, attempts to offset mephedrone-induced anxiety through the consumption of *Cannabis* (Zawilska & Wojcieszak, 2013; Lopez-Rodriguez & Viveros, 2019) may serve to elicit other adverse effects such as delusions and hallucinations, if not exacerbation of other adverse effects evoked by mephedrone (Lopez-Rodriguez & Viveros, 2019).

1.4.6.1 Acute effect on anxiety

Pre-clinically, the elevated plus maze (EPM) (Walf & Frye, 2007) and open-field arena (Prut & Belzung, 2003) are tests which aim to assess anxiety in rodents, based on their aversion to open spaces, and thigmotaxic tendency.

Acutely, (*S*)-mephedrone (300.0 µM, dissolved in spring water in a Petri dish) has been shown to produce an anxiolytic effect in planaria akin to that following fluoxetine (1.0 µM) or ethanol (1%) (Zewde *et al.*, 2018), as well as in rats (10.0 or 30.0 mg kg^{-1}, i.p.) 48 h following cessation of either cocaine or MPDV binge regimens (10.0 or 1.0 mg kg^{-1} respectively, three times per day for ten days, one hour intervals), manifest in improvements in forced swim test (a test of depression-like behaviours) and EPM performance (Philogene-Khalid *et al.*, 2017). Such an effect has also been noted 30 min following high dose mephedrone (30.0 mg kg^{-1}, i.p.) in female C57BL-6 mice, as illustrated by an increase in time spent in open arms and exploratory head dips on the EPM (Pail *et al.*, 2015). In contrast, repeated administration at a lower dose (10.0 mg kg^{-1}, i.p., on days one, two, and eight) elicited an anxiogenic profile in Lister-Hooded rats (Shortall *et al.*, 2016a). However, neither augmentation was noted in C57BL/J6 mice 14 days following repeated administration at a high dose (30.0 mg kg^{-1}, i.p., twice daily, four consecutive days) (den Hollander *et al.*, 2013), indicating a capacity for mephedrone to effect acute, but not prolonged, alterations in anxiety-related behaviours, though in no conclusive direction. Alternatively, this suggests the anxiolytic effect of mephedrone is exclusive to rats (in the cases aforementioned).

The emission of ultrasonic vocalisations (USVs) constitutes a form of social communication between rats. These USVs can be grouped into “aversive” or “prosocial” subtypes, which are emitted at 22 and 50 kHz, respectively. Further, prosocial calls can be subcategorised into three distinct types based on their specific pattern analysis (flat; step; and trill; the characteristics of which are described in further detail in Section 2.3.3.4) (Brenes *et al.*, 2016). Prosocial calls are widely

believed to be emitted in response to positive emotional states. Trill calls in particular are observed following acute administration of drugs such as MDMA, amphetamine and morphine (Wright *et al.*, 2010; Simola, 2015), whilst by contrast, distress calls are emitted in response to aversive stimuli which include predatory odours, foot shocks and bright lights (Portfors, 2007; Brudzynski, 2015). At present, no data are available on the effect of mephedrone on USV emission, although administration at a dose of 10.0 mg kg^{-1} (i.p.) has been shown to robustly increase distress call frequency in one-day-old domestic chicks (Zsedenyi *et al.*, 2014), indicating an aversive effect in this particular animal at this young age.

1.4.6.2 Mitigation of mephedrone-induced anxiety

In the study of Shortall et al., co-administration of caffeine (10.0 mg kg^{-1}, i.p.) served to reverse this mephedrone-induced anxiogenic profile, rendering anxiolytic behaviour in rats (Shortall *et al.*, 2016a). Complementing this, an online survey of recreational NPS users in Slovenia indicated 9.4% co-administered another drug or food substance in order to negate or offset NPS-related adverse effects (Sande, 2016), and indeed Shortall *et al.* suggest caffeine's propensity for this reversal as an incentive for recreational users to avert anxiety.

Other drugs frequently co-consumed with mephedrone in the recreational context include MDMA and nicotine. When co-administered at sub-threshold doses, mephedrone (0.05 mg kg^{-1}, i.p.) failed to augment the non-effect of MDMA (0.1 mg kg^{-1}, i.p.) on EPM behaviours of male Swiss mice, which exhibited robust anxiogenic behaviours following MDMA at higher doses (Budzynska *et al.*, 2017). In contrast, co-administration of nicotine (s.c.) and mephedrone (i.p.) at sub-threshold doses (0.05 mg kg^{-1}) effected a significant anxiogenic profile in male Swiss mice (Budzynska *et al.*, 2015).

1.4.6.3 Long-term effect of mephedrone on anxiety

Repeated and intermittent MDMA administration is evidenced to cause anxiogenic profiles of rodents on EPM ten days (Faria *et al.*, 2006), three weeks (Rodriguez-Arias *et al.*, 2011), and nine weeks (Gurtman *et al.*, 2002) later. However, absence of such changes have also been noted at eight days (Mechan *et al.*, 2002), three weeks (Daza-Losada *et al.*, 2008), 29 days (Mechan *et al.*, 2002) and 59 days (Bull *et al.*, 2004), though the work of Mechan *et al.* documented anxiogenic profiles at later time points, indicated by open field (73 days) and EPM (80 days) tests. In contrast, some studies report anxiolytic profiles in 'Oncins France 1' mice three weeks following treatment across weekend-regimens (Rodriguez-Arias *et al.*, 2015), and co-administered cocaine during this regimen (Daza-Losada *et al.*, 2008). The long-term effect of mephedrone on anxiety remains to be established, and will be a matter of investigation in the present thesis.

1.4.7 Tolerance

The development of tolerance towards the physiological or subjective effects of a drug is indicative of its abuse liability. In an effort to "recapture" the subjective effects achieved following early experience(s) with the drug, recreational users may increase the dose taken, as has been reported by MDMA/Ecstasy users (Verheyden *et al.*, 2003; Parrott, 2005). Tolerance has also been cited by recreational users following chronic use of Khat (Nencini *et al.*, 1984; Wabe, 2011) and synthetic cathinones (Zawilska *et al.*, 2013), including MDPV (Andrabi *et al.*, 2015) and mephedrone (Winstock *et al.*, 2011a; Rácz *et al.*, 2012; Ribeiro *et al.*, 2012).

In rats, tolerance has been shown to be developed towards the behavioural effects of cathinone (Zelger & Carlini, 1980; Foltin & Schuster, 1982; Schechter, 1986; 1990; Schechter & McBurney, 1991), cathine (Pehek & Schechter, 1990; Schechter, 1990), methylone (Goldsmith *et al.*, 2019) and MDPV (Atehortua-Martinez *et al.*, 2019;

Schiavi *et al.*, 2020). Appropriately for their similar pharmacological profiles, cross-tolerance has also been demonstrated preclinically between cathinone, cathine and amphetamine (Zelger & Carlini, 1980; Foltin & Schuster, 1982; Foltin *et al.*, 1983; Schechter, 1990). Consistently, repeated administration of mephedrone has been suggested to elicit tolerance towards mephedrones serotonergic effect to depress intracranial self-stimulation in rats, indicating a potential for abuse following repeated administration (Suyama *et al.*, 2019).

Interestingly, early preclinical studies of psychostimulant tolerance suggest that the rate of development is in part dependent on behavioural factors (Schuster *et al.*, 1966). The development of tolerance towards the hypophagic effect of amphetamine (Carlton & Wolgin, 1971; Campbell & Seiden, 1973) and cocaine (Woolverton *et al.*, 1978) for instance, is enhanced in rats administered these stimulants prior to feeding sessions, relative to those administered similar concentrations of the drug post-feeding. Elsewhere, tolerance toward the hyperthermic effects of methylone have been shown to develop more rapidly in female rats, relative to males, evident in the first week versus fourth, respectively (Goldsmith *et al.*, 2019). MDMA-induced hyperthermia was thereafter converted to hypothermia in female rats by the fourth week of administration, though only blunted in male rats at this time point, a result attributed by Goldsmith *et al.* to differences in gene expression for heat generation in tissue measured via rectal probe.

These data collectively indicate that, akin to other psychostimulants and synthetic cathinones, and dependent on both context and usage pattern, tolerance (as opposed to sensitisation) towards the effects of mephedrone might be elicited in both recreational users and preclinical rodent models.

1.4.8 Cognition, learning and memory

In recreational users, mephedrone is indicated to acutely enhance psychomotor speed, perhaps through stimulation of dopamine receptors of the PFC and striatum. Following administration of a 200.0 mg dose, mephedrone has been shown to lessen alcohol-induced (0.8 g kg^{-1}) deficits in reaction times to neurocognitive testing (de Sousa Fernandes Perna *et al.*, 2016). However, anecdotal evidence suggests the co-administration of cannabis with mephedrone may elicit memory blackouts (Assi *et al.*, 2017), whilst co-use of synthetic cannabinoids impaired the driving ability of motorists in Northern Ireland (Cosbey *et al.*, 2013). Further, significant long-term deficits in semantic-, phonological- and working-memory have been noted in mephedrone users in states of sobriety (Freeman *et al.*, 2012; Herzig *et al.*, 2013).

1.4.8.1 Novel object discrimination

The novel object discrimination test, based on the innate preference of the rodent for novelty, assesses both attention and visual recognition memory (Antunes & Biala, 2012). Administration prior to task performance allows for the assessment of drug effects on this type of memory. Repeated, but not acute, administration of MDMA has been shown to impair NOD performance in mice (Nawata *et al.*, 2010), whilst impairments have also been noted following binge-type administration to rats (Rodsiri *et al.*, 2011). Importantly, adolescent rodents appear particularly vulnerable to such deficits following administration of MDMA in regimens mimicking recreational weekend use (review (Garcia-Pardo *et al.*, 2017)) and when delivered at high ambient room temperature (McGregor *et al.*, 2003). Interestingly, such a weekend regimen has been shown to effect concomitant decreases in hippocampal SERT binding two weeks later (Meyer *et al.*, 2008), whilst week-long repeated administration (10.0 mg kg^{-1}, i.p., once daily) has been shown to effect upregulation of hippocampal CB1 (cannabinoid type 1) receptors seven days, but not one day, later (Nawata *et al.*, 2010).

Prevention of such declines has been accomplished through pre-treatment with both memantine (Camarasa *et al.*, 2008), an NMDA and nicotinic receptor antagonist employed in the treatment of Alzheimer's Disease), and AM251 (a CB1 inverse agonist), as well as the genetic knockout of CB1 receptors (Nawata *et al.*, 2010), indicating MDMA's deleterious effects on NOD performance to be mediated through NMDA/nicotinic, and/or CB1 receptor activation.

Data concerning mephedrone's short- and long-term effect on NOD remain sparse. In one study, like cathinone and MDMA, daily i.p. administration of mephedrone across a two day period (4.0, 10.0 mg kg^{-1}) caused Lister-Hooded rats to spend significantly less time exploring novel objects in both familiarisation and choice trials on a NOD paradigm, though this rendered subsequent deficits in discrimination (after higher dose) subject to the interpretation that this was not attributable to deficits in recognition memory (Shortall *et al.*, 2013b). These impairments were replicated in a later study, and were not augmented by co-administration of caffeine (10.0 mg kg^{-1}) (Shortall *et al.*, 2016a).

In contrast, treatment across a four day regimen (i.p., twice daily, 6 h apart) with methamphetamine (5.0 mg kg^{-1}) or mephedrone (30.0 mg kg^{-1}) produced no changes in Wistar rats seven days following cessation of treatment (den Hollander *et al.*, 2014a), whilst impaired discrimination seven weeks after a longer dosing regimen (daily, 10 consecutive injections) was only noted following administration of mephedrone at a high (30.0 mg kg^{-1}) – and not lower (7.5, 15.0) – dose (Motbey *et al.*, 2012b). In all, these data indicate mephedrone to impair recognition memory acutely, though not chronically.

1.4.8.2 Spatial working memory

Preclinical data indicate repeated administration of mephedrone at high doses (25.0, 30.0 mg kg^{-1}) caused significant deficits in spatial working memory of rodents, as indicated by worsened performances on Morris Water Maze (MWM) and T-Maze tests (den Hollander *et al.*, 2013; Lopez-Arnau *et al.*, 2015; Ciudad-Roberts *et al.*, 2016a). Ciudad-Roberts *et al.* showed these deficits in MWM performance to be potentiated upon co-administration of ethanol. Of concern, mephedrone (10.0 mg kg^{-1}) has been shown to cross via the placenta and enter mouse foetus and brain (Strange *et al.*, 2017), and a regimen similar to that aforementioned (50.0 mg/kg, s.c., thrice x daily, two consecutive days per six days, for 18 days) has been shown to produce similar deficits, accompanied by hippocampal damage, in offspring of Balb/C mice (Naseri *et al.*, 2018). This raises concern for the use of mephedrone in pregnant human users, and an analytical method has been recently developed and validated which allows for the detection of mephedrone in meconium samples of the unborn foetus (López-Rabuñal *et al.*, 2019; Nemeškalová *et al.*, 2019).

No study to date has examined mephedrone's effect on spatial memory and discrimination via the novel object location (NOL) paradigm; a paradigm on which performance is demonstrably susceptible to improvement or decline dependent on drug administration (Umka Welbat *et al.*, 2016; Welbat *et al.*, 2016a; Welbat *et al.*, 2016b), including seven days following repeated administration of methamphetamine to mice (Chiu *et al.*, 2014).

1.4.8.3 Memory consolidation

Memory consolidation has also received little investigative attention. Consistent with the known effect of psychostimulants to improve cognition upon acute administration, the co-administration of mephedrone (2.5 mg kg^{-1}) with subthreshold doses of MDMA (1.0 mg kg^{-1}) (Budzynska *et al.*, 2017) or nicotine (0.05 mg kg^{-1}) (Budzynska *et al.*, 2015),

robustly facilitates memory consolidation (of a paired foot shock) in male Swiss mice (indicated by increased index latency) on a Passive Avoidance paradigm.

1.4.8.4 Hippocampal- and amygdala-related associative memory

The conditioned emotional (or freezing) response (CER/CFR) is a test measuring amygdala and associative memory. Performance on this task is impaired by the amphetamines (Cappell *et al.*, 1972), whilst associative memory for the context, but not the aversive cues, has been shown to be disrupted in Lister-Hooded seven days following cessation of repeated mephedrone administration, indicating hippocampal, but not amygdala-related impairment (Shortall *et al.*, 2013b).

Elsewhere, mephedrone's capacity to augment performance of Rhesus Macaque monkeys on a visuospatial paired-associate learning task was contingent on dose employed and task difficulty, whereby 0.32 mg kg^{-1} (i.m.), but not higher or lower doses, rendered enhancements in performance on the highest difficulty mode of the task. Lowering of task difficulty also impeded performance. Interestingly, administration of *d*-methamphetamine at similar doses rendered similar effects, with improvements only observed at a dose of 0.32 mg kg^{-1} (Wright *et al.*, 2012). The authors acknowledged that such commonalities between the dose-dependent effects of the substances tested were consistent with the Yerkes-Dodson law, whereby doses precipitating improvements in performance might aptly facilitate physiological arousal optimal for task performance.

1.4.9 Sensorimotor gating

Sensorimotor gating refers to the inhibition of the startle reflex as a motor response to a sensory event, as a consequence of prior exposure to a relatively weaker sensory

event. Prepulse inhibition (PPI) is a measure of sensorimotor gating in which the startle reflex is blunted by a weak sensory event (referred to as a prepulse), and is employed in preclinical rodent models to study the potential modifying effect(s) of experimental manipulation. It is posited that PPI is regulated by limbic cortico-striato-pallidopontine (CSPP) circuitry, and is impaired in a variety of neuropsychiatric disorders, including schizophrenia, obsessive compulsive disorder and Tourette's syndrome (Swerdlow *et al.*, 2016).

Aside from neuropsychiatric conditions, PPI in rats can be disrupted following pharmacological manipulation, and indeed is impaired upon acute administration of MDMA (Kehne *et al.*, 1996b; Vollenweider *et al.*, 1999). Long-term impairment has also been noted in rats 39 days following cessation of repeated administration of MDMA during adolescence, with greater inhibition noted in animals co-administered THC throughout this time (Llorente-Berzal *et al.*, 2013).

To date, PPI has been shown to be unimpaired in rats by mephedrone alone (Shortall *et al.*, 2013b; Sichova *et al.*, 2017) or with caffeine (Shortall *et al.*, 2016a). However, no study has examined the long-term effect of mephedrone on PPI in adulthood, following repeated exposure during adolescence. This shall be examined in the first experiment of the present thesis.

1.5 Experimental aims of this thesis

The methodological techniques employed in the present thesis aim to assess the acute effects of mephedrone on body temperature, locomotor activity, anxiety-like behaviour and stereotyped behaviours, as well as any modification of these effects by repeated administration and/or co-administration of caffeine. Further to this, lasting cognitive effects of these drugs both independently and in combination are to be assessed through the use of various behavioural tests, specifically NOD (to test recognition memory), EPM (to test anxiety), PPI (to test sensorimotor gating) and CFR

(to test associative memory), as well as the determination of lasting effects on hippocampal microglial activation.

A separate study will seek to elucidate the pharmacological mechanism by which caffeine and mephedrone, in combination, elicit the behavioural changes observed in the first experiment. This second experiment will entail the collection of striatal dopamine and 5-HT content via *in vivo* microdialysis, with analysis by HPLC-ED, and will again ascertain drug-induced changes in body temperature, locomotor activity and stereotyped behaviours.

Chapter 2. Acute and longer lasting effects of repeated binge dose administration of mephedrone, with and without caffeine

2.1 Introduction

As outlined in Chapter 1, the acute effects of mephedrone in rats include hypothermia (Section 1.4.2), locomotor hyperactivity (Section 1.4.1), anxiety-like behaviour (Section 1.4.6), and the elevation of extracellular monoamine levels in several brain regions (Section 1.2.2). Although these effects do not appear to escalate with rapid repeated ('binge-type') administration (Shortall *et al.*, 2016b), they are modified by caffeine co-administration (Shortall *et al.*, 2016a). This finding mirrors those with other stimulant drugs that modulate monoamine efflux, including MDMA (Ikeda *et al.*, 2011; Gorska & Golembiowska, 2015; Gorska *et al.*, 2018).

In determining the research aims of the present chapter, there appeared to be a notable deficit in the preclinical literature pertaining to the modifying effect of caffeine on the effects of mephedrone, save for one previous study of this group. Given the known co-use of caffeine with mephedrone by recreational users, it was deemed prudent to mitigate this deficit by investigating this combination. In order to mimic the recreational use of this drug combination, a repeated administration or ('binge-type') regimen was employed, whereby the effects of multiple daily administrations of drug(s) on adolescent rats were observed on two consecutive days per week across two successive weeks. Subsequent assessment of lasting effects of these administrations on cognition, aversion and hippocampal microglial activation were made in these same rats in adulthood. The study is described herein.

In addition to acute cognitive deficits in rats (den Hollander *et al.*, 2013; Lopez-Arnau *et al.*, 2015; Ciudad-Roberts *et al.*, 2016b), there is some evidence that repeated mephedrone administration induces lasting memory deficits after drug washout. For example, impaired recognition memory has been noted seven weeks following repeated high dose administration (Motbey *et al.*, 2012b) (Section 1.4.8.3), whilst selective deficits have been observed in hippocampal-dependent contextual association following mephedrone administration (Shortall *et al.*, 2013b) (Section 1.4.8.4). However, in the study of Motbey *et al.*, recognition memory was not impaired

following low dose administration (Motbey *et al.*, 2012b), nor seven days following administration across a shorter dosing regimen elsewhere (den Hollander *et al.*, 2014a). Similarly, Shortall *et al.* observed no acute deficits in hippocampal- and amygdala-dependent cued association following mephedrone administration (Shortall *et al.*, 2013b), and no long-term changes in anxiety-like behaviour have been noted following repeated administration in mice (den Hollander *et al.*, 2013). At present, no data is available on the lasting effect of mephedrone on sensorimotor gating, though impairments have been observed 39 days following repeated administration of MDMA during adolescence (Llorente-Berzal *et al.*, 2013) (Section 1.4.9).

Caffeine, aside from its role as adulterant in 'legal high' products (Brandt *et al.*, 2010b; Davies *et al.*, 2010; Zuba & Byrska, 2013; Peterfi *et al.*, 2018), is present at relatively high doses in energy drinks (Howard & Marczinski, 2010) such as Red Bull®, which are often consumed by adolescents and young adults (Curran & Marczinski, 2017), amongst other purposes, to combat fatigue and drowsiness resultant of MDMA (Kaminska *et al.*, 2018) or alcohol (Pennay & Lubman, 2012) consumption. In rats, caffeine has been shown to exacerbate neurotoxicity induced by the amphetamines (Frau *et al.*, 2013), including MDMA (Vanattou-Saifoudine *et al.*, 2012), and potentiate MDMA-induced microglial and astroglial activation in mouse striatum (Khairnar *et al.*, 2010), whilst the effect of caffeine in combination with mephedrone on measures of neurotoxicity remains to be investigated at the time of writing. Importantly, caffeine's action to increase extracellular levels of 5-HT content (Nehlig *et al.*, 1992), as well as to enhance MDMA-induced 5-HT efflux (Gorska & Golembiowska, 2015) via adenosine receptor antagonism, may constitute an adverse effect for users co-administering mephedrone and caffeine. This is because sufficient elevation of synaptic 5-HT content, such as by co-administration of multiple serotonergic drugs, is known to precipitate the serotonin syndrome (Bartlett, 2017), which has been reported previously in a mephedrone user with a medical prescription for fluoxetine (Garrett & Sweeney, 2010).

Acute co-administration of caffeine (10.0 mg kg^{-1}, i.p.) has previously been shown by this group to enhance mephedrone-induced (10.0 mg kg^{-1}, i.p.) locomotor activity, convert mephedrone-induced hypothermia to hyperthermia, and prevent mephedrone-induced anxiogenic behaviour (Shortall *et al.*, 2016a). However, HPLC-ED analysis determined repeated intermittent co-administration of caffeine and mephedrone did not precipitate neurotoxicity seven days post-cessation. Consistent with this, binge dose administration of mephedrone has elsewhere been shown to not produce lasting changes in striatal microglial or astroglial activation in mice (Angoa-Perez *et al.*, 2012; Angoa-Perez *et al.*, 2014). However, increases in glial fibrillary acidic protein (GFAP) immunoreactivity have been observed in the dentate gyrus of mice seven days following binge dose administration of mephedrone (3 x 25.0 mg kg^{-1}, s.c., two hours between doses, for two days) (Martinez-Clemente *et al.*, 2014).

Therefore, the current study examined the acute effects of repeated administration of mephedrone, with and without the co-administration of caffeine, on body temperature, the behaviour of the rat in the home cage, and open field test (OFT) activity during adolescence. A dosing regimen consisting of three injections per day, on two consecutive days per week for two weeks was employed, mimicking rapid re-dosing patterns of recreational use (Rodsiri *et al.*, 2011; Schifano *et al.*, 2011; Shortall *et al.*, 2016b) that are typically repeated over weekend periods. The dosing interval was determined as 2 h based on observations that mephedrone is rapidly taken up into and cleared from rat brain (Aarde *et al.*, 2013), and that peak concentrations are measured in plasma (Martinez-Clemente *et al.*, 2013; Miller *et al.*, 2013) and brain (Aarde *et al.*, 2013) within 1 h, and is an established protocol within our laboratory group (Rodsiri *et al.*, 2011; Shortall *et al.*, 2016b). Following an approximately three week period of drug absence, rats were tested in adulthood on a battery of tests of cognition and aversion, typically weekly, ordered from least to most aversive. Lasting changes in ambulatory activity and repetitive movement were assessed using a photobeam activity monitoring system (Dunphy-Doherty *et al.*, 2018), hippocampal-dependent recognition memory was investigated by the NOD task (Antunes & Biala, 2012), lasting changes in anxiety were determined by the EPM task (Walf & Frye,

2007), alterations in sensorimotor gating were assessed by the PPI task (Swerdlow *et al.*, 2016), and hippocampal- and amygdala-dependent associative memory were gauged by the CFR task (Jones *et al.*, 2011). With the exception of CFR, none of these tasks warranted training, but rather relied on the natural instinct of the rat. Rats were subsequently killed, and immunohistochemistry was performed to determine any changes in microglial activation within the hippocampus.

The dose of caffeine was selected from a previous study as this dose is known to produce changes in behaviour without any severe adverse effects (Shortall *et al.*, 2016a). We had intended to use an identical dose (10.0 mg kg^{-1}) of mephedrone but this ultimately proved impossible due to a supply chain issue. We had already delayed the planned start of the work by 16 weeks in an attempt to overcome this, but any further delay would have meant the author was unable to start this nine week long experiment before the third month of the second year of PhD study. We therefore proceeded with a lower dose of 7.5 mg kg^{-1}, which was deemed appropriate because repeated administration at lower doses (5.0 mg kg^{-1}, 4 consecutive days per week for 2 weeks) has been shown to precipitate long-term neurotoxicity in Wistar-Han rats (Kaminska *et al.*, 2018).

Despite the previous observation that co-administration of caffeine and mephedrone caused hyperthermia up to 2 h post-administration (Shortall *et al.*, 2016a) these measurements were obtained via rectal probe – which constitutes a more invasive and thus more stress-inducing procedure (Dallmann *et al.*, 2006) – and animals were housed in grid top cages. Technical refinements aimed at improving animal welfare determined, for the present study, the use of subcutaneous (s.c.) temperature chips, allowing for minimally-invasive scanning for this measurement, and the housing of animals in individually ventilated cages.

2.2 Aims

The aims of this study were to:

(1) determine the acute effects of binge dose administration of mephedrone and caffeine, individually and in combination, on body temperature, anxiety-like behaviour, locomotor activity and stereotyped behaviour in adolescent rats;

(2) probe the long-term effects of these drug administrations on locomotor activity, recognition memory, anxiety-like behaviour, sensorimotor gating and fear-associative memory in adulthood;

(3) elucidate any lasting alterations in microglial activation within the hippocampus in adulthood.

2.3 Materials & Methods

2.3.1 Animals

Forty experimentally-naïve adolescent male Lister hooded rats postnatal day (PND) 20/22/23/24 (n = 10 of each age) upon delivery (Charles River UK) were housed in groups of 3 – 4 per cage on a 12 h light-dark cycle (lights on at 0700 hrs) in individually ventilated cages (GR1800 double-decker; Techniplast, UK) containing sawdust bedding, wooden blocks and cardboard tubes for environmental enrichment. Ambient temperature (21.0 ± 1.0^{o}C) and relative humidity (60 ± 5%) were constant, food (Teklab Global Rodent Diet®, Envigo, UK) and water were available *ad libitum*. All experiments were conducted during the light phase (09:00 – 17:00 h). Selection of the Lister hooded strain was predicated on the relatively increased activity seen in this strain during the light phase, compared with other strains (McDermott & Kelly, 2008), and to maintain consistency with previous work of our group (Shortall *et al.*, 2013a; Shortall *et al.*, 2013b; Shortall *et al.*, 2016a; Shortall *et al.*, 2016b). The selection of male rats was also predicated on this latter reason, as well as the greater incidence of mephedrone use in male, relative to female, recreational drug users. Doses of drugs were selected to

comply with the 3 Rs of humane animal testing. All experiments were conducted in accordance with the Animal (Scientific Procedures) Act, 1986, and ARRIVE guidelines (Kilkenny *et al.*, 2010; Percie du Sert *et al.*, 2019), with approval from the University of Nottingham Local Ethical Committee. Body weight was recorded immediately prior to first injection of each dosing day, and at intervals of no more than weekly thereafter, to ensure no rat lost 20% of its own body weight, consistent with the moderate severity band permitted by this groups Home Office Project Licence. Blinding was achieved by one experimenter preparing drug solutions and labelling them with a code, before providing to a separate experimenter who performed the injections. Data were acquired by further experimenters with no knowledge of either treatment identity or treatment code.

2.3.2 Drugs

(±)-mephedrone-HCl was purchased from Sigma-Aldrich and Ascent Scientific (with the drug purity of each provider being equivalent). Caffeine ReagentPlus was obtained from Sigma-Aldrich. All drugs were dissolved in saline vehicle (0.154 M). Doses are quoted as the salt.

2.3.3 Experimental design

Rats (n = 10 per treatment group, consistent with previous mephedrone studies of this group) received i.p. injections of saline vehicle (1.0 mL kg^{-1}), (±)-mephedrone-HCl (7.5 mg kg^{-1}), caffeine (10.0 mg kg^{-1}), or a combination of (±)-mephedrone-HCl and caffeine thrice daily (two hours between each injection) on two consecutive days a week for two weeks (days one, two, eight and nine of the experiment; **Fig. 2.1**), with behavioural testing to evaluate lasting effects on LMA (PND 59-63), NOD (PND 61-64), EPM (PND 67-71), PPI (PND 74-78), CFR (PND 81-85). One day following CFR testing (PND 82-86), brain tissue was collected for immunohistochemical analyses.

The number of animals and duration required to assess each individual necessitated testing to be split over multiple days, and care was taken to ensure a mix of treatment combinations across days. All apparatus were cleaned with 20 % ethanol prior to use and between animals to remove any olfactory cues.

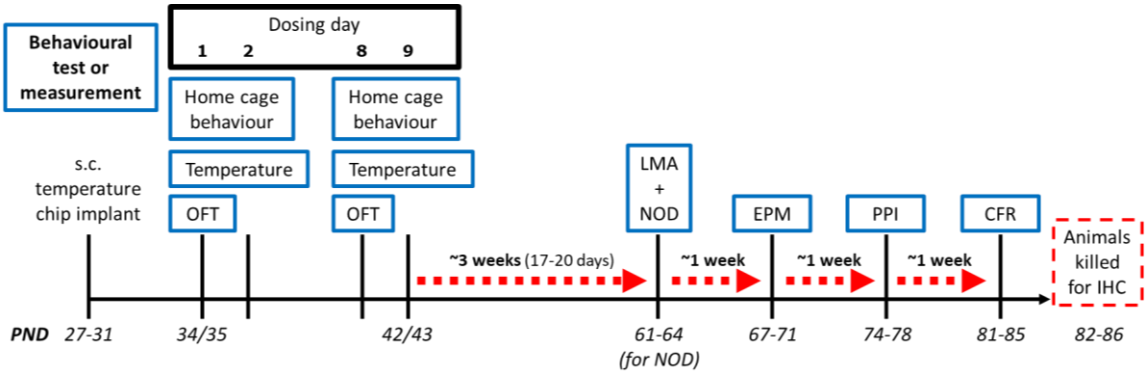


Figure 2.1 Experimental design. Adolescent male Lister hooded rats (n = 10 per treatment group) received i.p. saline vehicle (1.0 mL kg^{-1}), mephedrone (7.5 mg kg^{-1}), caffeine (10.0 mg kg^{-1}) or mephedrone and caffeine thrice daily (2 h apart) on two consecutive days a week for two weeks. On all dosing days, body temperature and behaviour in the home cage were measured. Open field exploration was measured on the first day of each dosing week. Following a washout period (17 – 20 days), tests were conducted on an approximately weekly basis: locomotor activity (LMA) and novel object discrimination (UNODC) (approx. three weeks post-final injection); elevated plus maze (EPM) (approx. four weeks post-final injection); prepulse inhibition (PPI) (approx. five weeks post-final injection); and conditioned freezing response (CFR) (approx. six weeks post-final injection). Rats were killed 24 h following CFR retention testing.

2.3.3.1 Temperature recording

On PND 27 – 31 rats were non-surgically implanted with subcutaneous temperature-sensing microchips into the nape of the neck (with Bio-Thermo® idENTICHIP, Animalcare; York, UK) **(Fig. 2.2)** to allow subsequent physiological monitoring in freely-moving animals with minimal disturbance. The use of a microchip rather than a rectal probe allows more regular measurement of body temperature without restricting locomotor activity or triggering stress-induced hyperthermia and therefore represents a clear refinement, in terms of both animal welfare and data quality, where multiple temperature readings are required. In-house validation studies confirmed that basal temperatures detected via s.c. microchips did not differ from those provided by i.p. telemetry monitors in the same rats, and show that microchips were able to detect drug-induced hyperthermia (Shortall et al., unpublished observations) and hypothermia (Kohli *et al.*, 2019; Goh *et al.*, 2020). Body temperature was recorded via a handheld scanner immediately prior to, and at 15 min intervals for 60 min after each injection. It was also measured immediately before and after EPM and CFR testing.

Figure 2.2 Microchip and scanner used to measure body temperature throughout experiment. On PND 27 – 31, adolescent male Lister hooded rats (n = 40) received non-surgical subcutaneous implantation of a temperature-sensing microchip (Bio-

Thermo® idENTICHIP, Animalcare; York, UK) into the nape of the neck. This allowed subsequent physiological monitoring in freely-moving animals with minimal disturbance.

2.3.3.2 Stereotyped behaviour

The use of researcher-devised numerical scales for the measurement of stereotyped behaviour in response to drug administration in rodents has been established for decades (Creese & Iversen, 1973; Ellinwood & Balster, 1974; Setler *et al.*, 1978; Sturgeon *et al.*, 1979; Havemann *et al.*, 1986; Harkin *et al.*, 2000; 2001a; 2001b). Fifteen min post-injection, home cage behaviour of rats was observed for a period of 10 s without interruption of the animal, and scored on a scale of values ranging 0 to 15 **(Fig. 2.3)**. This scale was devised by the experimenter, and resultant score reflected the most severe single behaviour observed (i.e. the peak activity, and not a cumulative total).

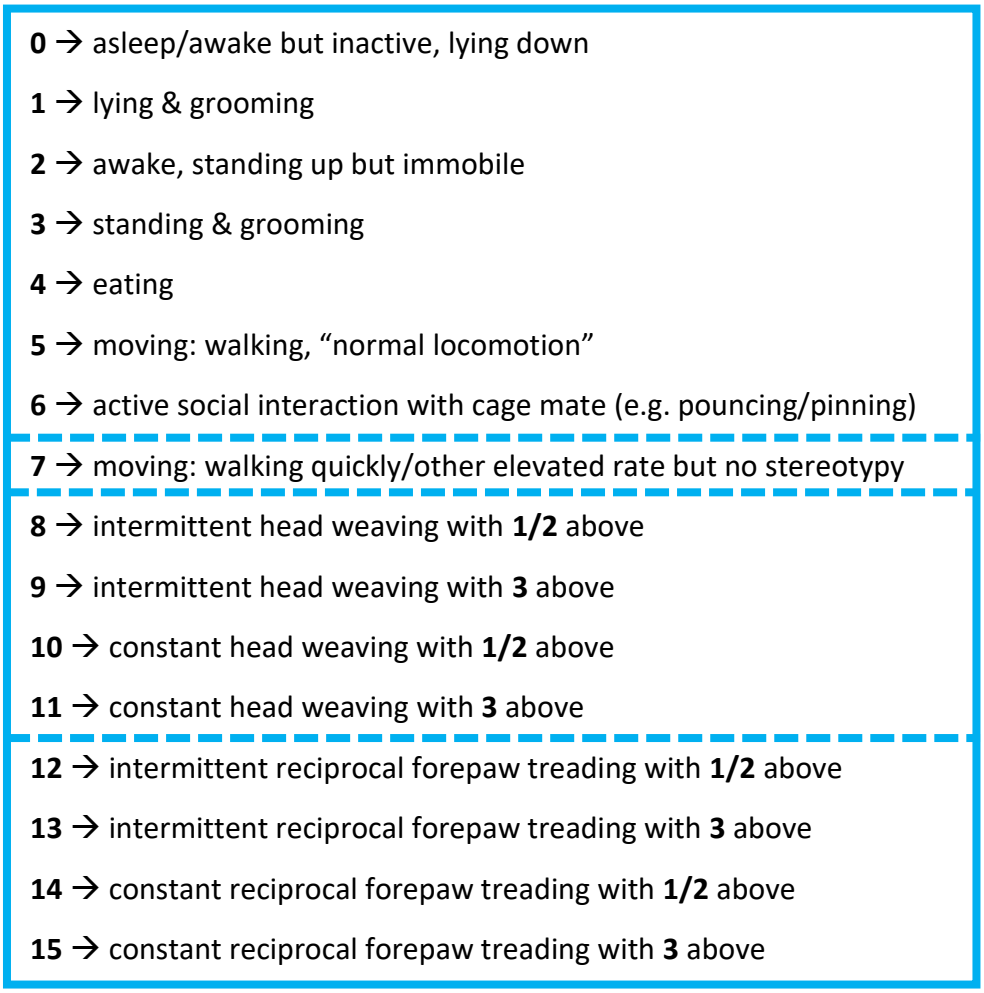


Figure 2.3 Behavioural rating scale. Behaviour of animals in the home cage was observed 15 min following each injection and scored on a numerical scale of 0 to 15 (0 to 6: normal home cage activity and social behaviour; 7: hyperactivity without stereotypy; 8 to 11: increasing severity of lateral head weaving; 12 to 15: increasing severity of reciprocal forepaw treading).

2.3.3.3 Open field test

Thirty min following the first injection of each week (days 1 and 8, see **Fig. 2.1**), rats were individually placed into an open field arena (75 cm diameter, 45 cm wall height) **(Fig. 2.4)** as described previously by our group (Dunphy-Doherty *et al.*, 2018) lined with grey card, at 60 lux, and activity was recorded for 15 min by Ethovision XT7 software. Distance moved (cm), average velocity (cm s^{-1}), duration (s) in zones (central and

peripheral; for definition of these zones, see **Fig. 2.4**) and entry frequencies of each zone were computed by this software. Grooming and rearing (supported and unsupported; i.e. with or without the use of the arena wall) was recorded by an independent observer. The number of faecal pellets were counted.

Figure 2.4 Open field arena used to assess acute drug-induced behavioural changes. On days one and eight, adolescent male Lister hooded rats (n = 10 per treatment group) received i.p. saline vehicle (1.0 mL kg^{-1}), mephedrone (7.5 mg kg^{-1}), caffeine (10.0 mg kg^{-1}) or mephedrone and caffeine. Thirty minutes later, they were placed in the open field arena for 15 minutes, to assess drug-induced changes in locomotor activity and anxiety-like behaviour.

2.3.3.4 Ultrasonic vocalisations

The capture and analysis of distress/alarm and prosocial USVs of rats is an established technique of our group (Watson *et al.*, 2016; Kohli *et al.*, 2019; Goh *et al.*, 2020). During the open field test, USVs were recorded via an electret microphone (Emkay, Avisoft Bioacoustics, Germany) connected to an ultrasound detection unit (Ultrasound Gate, customised model 112, Avisoft Bioacoustics, Berlin). Recorded signals were digitalised and saved as .wav files. Using Avisoft analysis software (SAS-Lab Pro, v. 4.38, Avisoft Bioacoustic Berlin), recordings were truncated to coincide with time spent in the open

field arena, temporal and frequency characteristics of calls were extracted, and spectrographs were produced using the following parameters: 488 Hz frequency resolution; 512 FFT (Fast Fourier transformation) length; 75% frame size; and 93.75% temporal resolution overlap. Lower and higher cut-off frequencies of 22 and 85 kHz, respectively, were used to reduce background noise. Various parameters, including peak amplitude, peak frequency, minimum and maximum bandwidth, were automatically detected. The total number of USVs was calculated and USVs were grouped into aversive (22 kHz) or prosocial (50 kHz) subtypes, based on parameters of both bandwidth and spectrograph. Further, 50 kHz calls were grouped into three categories based on their specific pattern analysis: flat; step; and trill (Brenes *et al.*, 2016). Definitions of these categories were as follows:

- Flat: a call with peak frequency changes less than or equal to 5 kHz. However, the difference between start and end frequency could exceed 5 kHz if the spectrogram representation of the call is displayed in an ascending or descending direction **(Fig. 2.5A)**;
- Trill: a call with one or more frequency modulations containing an element longer than those present in step, and/or displaying a zig-zag shape which may appear as a series of inverted-U shapes **(Fig. 2.5B)**;
- Step: a flat call with one or more short element at least 5 kHz lower or higher than the principal call **(Fig. 2.5C)**.

Calls not fitting into any of these three categories were removed from analysis (34 and 1, in weeks 1 and 2 respectively). Example spectrographs are provided **(Fig. 2.5)**. For each call subtype (flat; step; trill), differences in total number of calls emitted were determined by a log10 transformation followed by two-way ANOVA analysis.

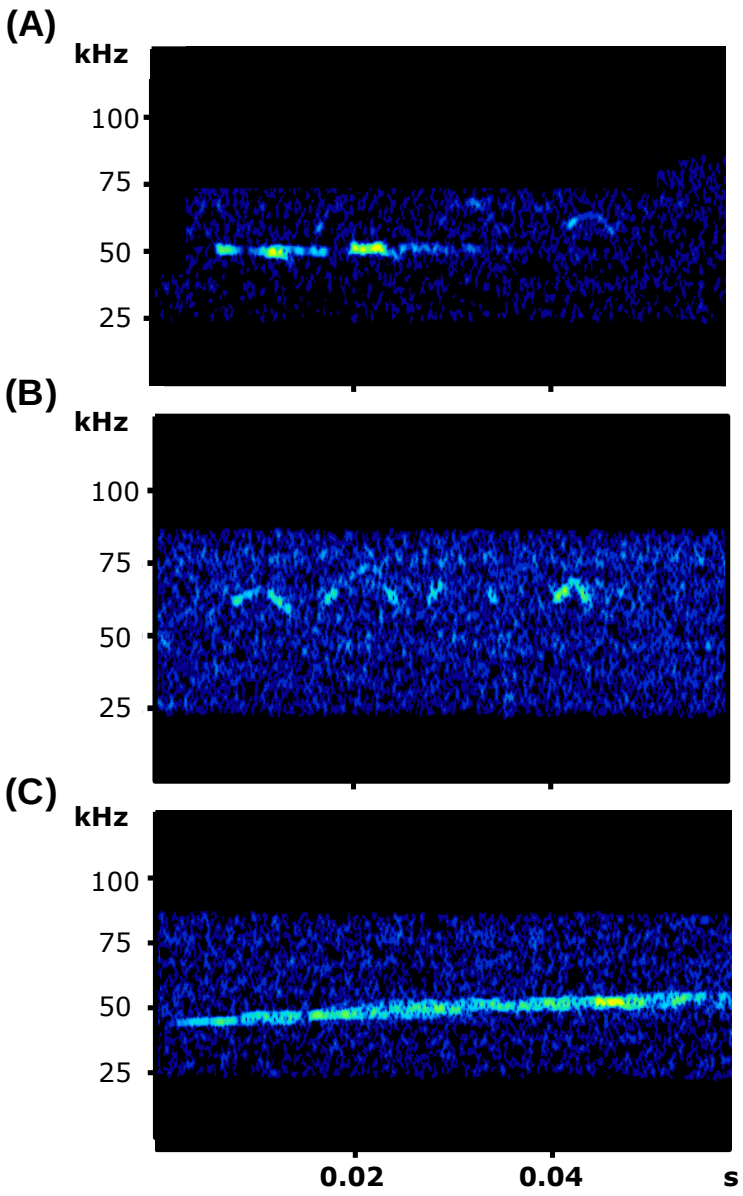


Figure 2.5 Example spectrograph images of ultrasonic vocalisations. Spectrographs show prosocial 50 kHz calls (x axis time scales in seconds) categorised into **(A)** flat, **(B)** trill and **(C)** step calls, according to pattern analysis.

2.3.3.5 Locomotor activity

LMA was assessed on PND 59/61/62/63 during a 1 h period in unfamiliar individual Perspex arenas (39.0 x 23.5 x 24.5 cm) with wire mesh lids. Ambulation, rears and fine movement were recorded via a Photobeam Activity System (San Diego Instruments,

CA) comprising a lower and upper layer of infra-red beams, as described in a previous study of this group (Jones *et al.*, 2011). Activity counts were differentiated into total ambulation (consecutive breaking of two adjacent lower level beams recorded a single count for fine movement, and breaking of a single upper level beam recorded a single count for rearing).

2.3.3.6 Novel object discrimination

In the same arena as used for LMA **(Fig. 2.6)**, NOD was assessed on PND 61/63/64 to determine any lasting effects of drug treatment on hippocampal-dependent recognition memory, with an established protocol of our laboratory (King *et al.*, 2004; King *et al.*, 2009; Watson *et al.*, 2012; McIntosh *et al.*, 2013; Shortall *et al.*, 2016a). Twenty four or 48 h following LMA assessment, rats were returned to the same individual arena to habituate for a further 3 min. This was followed by 1 min in the home cage, and two consecutive 3 min object exploration trials, separated by a 2 h inter-trial-interval (ITI) in the home cage. In the first (familiarisation) trial, rats encountered two identical objects (cylindrical plastic bottles (8 cm height; 5 cm diameter) covered in white electrical masking tape, filled with water). In the second (choice) trial, one object was replaced by another object identical in height, diameter, and content, with the addition of four horizontal stripes of black electrical insulation tape. Objects were placed at the front left and back right of the arenas, 5 cm from the side and 10 cm from end wall, and the position of the novel object in the choice trial was pseudorandomised, such that five rats of each treatment group underwent the task with the novel object at the front left, whilst the object was placed at the back right for the remaining five rats.

Active exploration of each object – defined as sniffing, licking, chewing or having moving vibrissae whilst directing nose towards and ≤ 1 cm from object – was recorded using separate stopwatches for each object. Climbing on objects in the absence of directed interest was not included in this measure. The actual time spent exploring

object in the choice trial was used to calculate discrimination ratio [novel/(total choice trial object exploration)], with a discrimination ratio > 0.5 indicating more time spent exploring novel, relative to familiar, object.

Figure 2.6 Activity boxes used to assess LMA and NOD.

2.3.3.7 Elevated plus maze

EPM exploration was assessed on PND 67/69/70/71 to determine any lasting effects of drug treatment on anxiety-like behaviour in a mildly aversive environment, using a modified version of a previously described protocol of our group (Bull *et al.*, 2003; Shortall *et al.*, 2016a). The black Perspex maze comprised four arms at right angles around a central square (15 x 15 cm), elevated 90 cm from the floor **(Fig. 2.7)**. Two arms were "closed" (45.5 x 15.0 cm), with 42.5 cm high walls, and two arms "open" (46 x 16.5 cm) with no walls. Rats were placed in the centre of the maze, facing the intersection of a closed and open arm. Light intensity was ~ 18 lux in closed arms and ~ 60 lux on open arms. Animals were left for 5 min on the EPM, and exploration was recorded by Ethovision XT7 software [however, due to a technical error, exploration was recorded for 280 s (4 min 40 s)]. Number of arm (closed; open) entries, total time spent in each set of arms, and the percentage time in open arms ([time spent in open arms/time spent in all arms] x 100) were measured. Body temperature was measured immediately before and after testing. Manual scoring of frequency of unprotected head dips (over the sides of open arms) and protected stretch attends was also

conducted from video recordings by an independent observer. The number of faecal pellets were counted.

Figure 2.7 EPM used to assess anxiety-related behaviour.

2.3.3.8 Pre-pulse inhibition

PPI was measured on PND 74/76/77/78 to assess any long-term effects of drug treatment on sensorimotor gating, using an established protocol of the lab group (Jones *et al.*, 2011; McIntosh *et al.*, 2013; Shortall *et al.*, 2013b; Shortall *et al.*, 2016a). Four SR-lab startle response chambers (San Diego Instruments, CA) were used. Each contained a clear Perspex cylinder (8.8 x 19.5 cm) atop a piezoelectric transducer, all contained within a sound-attenuating individually-ventilated chamber (39 x 38 x 58 cm) **(Fig. 2.8)**. A test session consisted of 5 min acclimatisation to background white noise (62 dB), followed by ten successive startle alone pulses (120 dB), then by fifty startle trials (ten without pre-pulse and ten of each startle preceded by a 72 dB, 76 dB, 80 dB and 84 dB pre-pulse, randomised automatically by the software with unpredictable inter-trial intervals) and ending with five startle-alone pulses. Whole body startle responses were recorded every 1 ms across a 100 ms period, commencing from the initiation of the startle pulse, by Startle Reflex Testing Software (San Diego Instruments, CA), calculating a total cumulative area under the curve (AUC) response. Results are expressed as percentage PPI from average AUC for each trial type

(employing a conditional statement of eliminating any extreme values ± 2 SD from mean, which may be consequential of rat movement during startle delivery) using equation % PPI = [((pulse alone AUC – prepulse AUC)/pulse alone AUC) x 100].

Figure 2.8 SR-lab startle response chamber used to assess PPI.

2.3.3.9 Conditioned freezing response

CFR was measured on PND 81/83/84/85 to assess any long-term effects of drug treatment on associative memory, using an established protocol (Jones *et al.*, 2011; Woods *et al.*, 2012; McIntosh *et al.*, 2013; Shortall *et al.*, 2013b; Shortall *et al.*, 2018). Apparatus comprised a two-compartment box (25 x 25 x 27 cm internally; Panlab S-Lab, Spain) of one white-walled and one black-walled chamber, with Perspex door fronts, separated by a computer-operated door (8 x 8 cm) **(Fig. 2.9)**. Each chamber comprised a grid floor connected to a shuttle box control unit, with a light and centrally located speaker. On conditioning day (PND 80/82/83/84), body temperature of rats was recorded before being placed individually in the light compartment and allowed 30 s before the separating door was opened. Latency to enter the dark chamber was recorded, and the door was closed immediately following entry. After 30 s (habituation) in the dark chamber, rats were presented with a 5 s light (200 lux) and tone (89 dB, 3 kHz, conditioned stimulus (CS)) accompanied in the final 1 s by a foot shock (0.4 mA, unconditioned stimulus (US)). This light-tone-shock (CS-US)

combination was repeated twice more, with onset at 1 min intervals. Rats were removed from the dark compartment 1 min following the final such combination, body temperature measured, and returned immediately to the home cage. Duration of freezing (defined as absence of movement except that necessary for respiration) was measured using stopwatches for each 55 s period following cessation of the foot shock, allowing assessment of acquisition of the association between CS and US.

Twenty four hours later (PND 81/83/84/85), temperature was measured and rats were returned to the dark compartment. Freezing duration was measured over a period of 5 min (in absence of any cue or foot shock), allowing a measure of contextual fear-associated learning and memory. The light-tone combination (without foot shock) were then presented for 5 s, and freezing duration was again measured over the following 5 min period, assessing cued fear-motivated associative learning and memory. Rats were removed from the apparatus, temperature measured, and placed back in the home cage. The number of faecal pellets were counted.

Figure 2.9 Two compartment box used to assess CFR.

2.3.3.10 Tissue collection and neurochemical detection by IHC

The immunohistochemistry procedure was performed at room temperature except where stated otherwise. Rats were killed one day following CFR retention testing (PND 82/84/85/86) (which was equivalent to between 40 and 43 days after the final drug injection, **Fig. 2.1**) by concussion followed by immediate decapitation. Hemi-brains (39 left, 1 right) were rapidly dissected on a refrigerated table (BC72: Osborne refrigeration, UK, 4.0 ^{o}C) and immerse-fixed in 4% paraformaldehyde at 4.0 ^{o}C for 24 h, before being cryopreserved in 30 % sucrose solution in 0.1 M phosphate buffered saline (PBS) for a further 24 h (4.0 ^{o}C), before being snap-frozen in isopentane on dry ice.

Dorsal hippocampi, determined with reference to a stereotaxic atlas (Paxinos & Watson, 1983), were sectioned (60 µm) in the coronal plane using a freezing microtome (Anglia Scientific) and stored in antifreeze solution (1:1:1.25 1,2-Ethanediol (ethylene glycol; Sigma-Aldrich, UK): 1,2,3-propanetriol (glycerol; Sigma-Aldrich, UK): 0.1 M PBS) at –20.0 ^{o}C. Sections were washed four times in 0.1 M PBS (5 min per wash) and non-specific background was blocked by incubating for 1 h at room temperature in 2% normal goat serum in day 1 buffer (0.1 M PBS containing 0.5% BSA, 0.3% Triton-X100, 0.3 M glycine). Sections were then incubated for 21 h at room temperature in the primary antibody (1:2000, anti-Iba1, rabbit polyclonal; Wako, cat. no. 019-19741) (FUJIFILM Wako Pure Chemical Corporation; Osaka, Japan) in day 1 buffer, washed three times in day 2 buffer (0.1 M PBS containing 0.15% BSA, 0.1% Triton-X100, 0.3 M glycine), incubated for 1 h at room temperature in the secondary antibody (1:500, Alexa Fluor 568 goat-anti rabbit Ig G), then washed twice in day 2 buffer, and twice in 0.1 M PBS. Sections were mounted on gelatinised slides and left to dry at 4.0 ^{o}C in the dark. In a humidified tray, slides were rinsed with 0.1 M PBS, counterstained for 30 s with DAPI nuclear stain (1:2000; Sigma-Aldrich, Darmstadt, Germany), washed twice with distilled water, mounted with DABCO fluorescent mounting medium and cover slipped, and left to dry at 4.0 ^{o}C in the dark. They were stored until being later imaged on a Nikon E200 microscope with SPOT Advanced software (Diagnostic Instruments

Inc., MI, USA). The number of Iba-1-positive cells (Iba-1 is a protein commonly used to indicate microglial expression) within CA1, CA3 and dentate gyrus were counted from x 20 images. Anatomical boundaries were determined with reference to a digital atlas (Kjonigsen *et al.*, 2011).

2.3.4 Statistical analyses

All statistical analyses were conducted using GraphPad Prism (v 7.03) or SPSS v 22 software. Data were checked for normality and homogeneity of variance before use of parametric tests. LMA, body temperature, NOD and PPI data were analysed using three-way repeated measures ANOVA, with caffeine treatment and mephedrone treatment as between-group factors, and time (LMA; body temperature), object (NOD) or pre-pulse amplitude (PPI) as within-group factors. Importantly body temperature change at the 0 min baseline time point (which had a mean and variance of zero) were not included in any statistical analyses. Choice trial discrimination ratio (NOD), EPM and CFR data were analysed using a two-way repeated-measures ANOVA, with caffeine and mephedrone treatment as between-group factors. Tukey's multiple comparisons post-hoc tests were conducted where appropriate. $P < 0.05$ was considered significant, and these data are presented as mean ± SEM. However, USV data were not normally distributed so were analysed using non-parametric Kruskal-Wallis tests (equivalent of a one-way ANOVA, used to determine statistically significant differences between three or more groups) with Dunn's multiple comparisons post-hoc tests. Data for behaviour of rats in the home cage were also not normally distributed, but due to the absence of a non-parametric equivalent for a three-way-ANOVA, this test was applied nonetheless. $P < 0.05$ was considered statistically significant, and data are presented as median ± interquartile range.

2.4 Results

2.4.1 Effects during treatment

2.4.1.1 Body weight

All rats gained weight throughout the experiment ($F_{(10, 360)}$= 3560.200, P < 0.001). This was not influenced by either drug alone (caffeine: $F_{(1, 36)}$ = 1.400, P = 0.244; mephedrone: $F_{(1, 36)}$ = 1.112, P = 0.299), or in combination (caffeine x mephedrone $F_{(1, 36)}$ = 1.440, P = 0.238) **(Fig. 2.10)**.

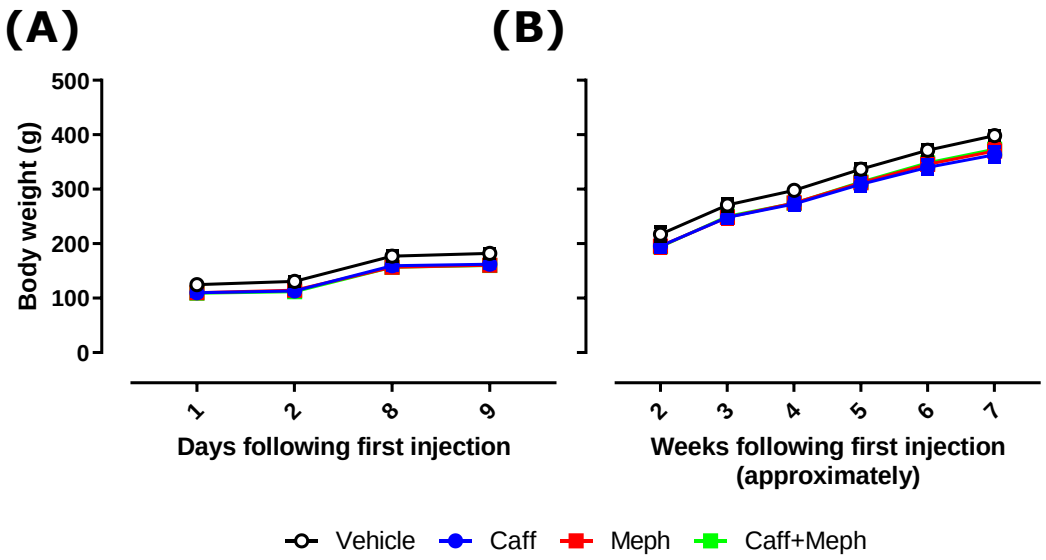


Figure 2.10 All rats gained weight irrespective of treatment. Body weight of male Lister hooded rats (n = 10 per treatment group) increased (mean ± SEM; error bars lie within symbols) across the duration of the experiment and was unaffected by repeated administration across days 1, 2, 8 and 9 during adolescence of vehicle, caffeine (Caff), mephedrone (Meph), or combination (Caff + Meph) **(A)**, or later in the approximately six week experimental period during adulthood **(B)**.

2.4.1.2 Changes in body temperature post-injection

There were no significant between-group differences in mean basal subcutaneous temperatures immediately prior to the first injection on each day, which were 37.48 ± 0.06 °C (day 1), 37.22 ± 0.05 °C (day 2), 37.23 ± 0.07 °C (day 8), and 36.98 ± 0.05 °C (day 9). These are consistent with other basal values using the same technique in our laboratory (e.g. 37.22 ± 0.10 °C; Goh *et al.* unpublished observations). The basal temperatures for each treatment group in each treatment day are shown in **Table 2.1**.

Table 2.11 Basal temperatures of all treatment groups on all treatment days.

Treatment group	Day			
	1	2	8	9
Vehicle	37.56 ± 0.15 °C	37.29 ± 0.10 °C	37.26 ± 0.10 °C	36.92 ± 0.13 °C
Caffeine	37.34 ± 0.15 °C	37.04 ± 0.12 °C	37.22 ± 0.16 °C	36.81 ± 0.13 °C
Mephedrone	37.58 ± 0.09 °C	37.23 ± 0.12 °C	37.30 ± 0.13 °C	37.07 ± 0.08 °C
Caffeine + Mephedrone	37.45 ± 0.08 °C	37.30 ± 0.07 °C	37.14 ± 0.13 °C	37.13 ± 0.06 °C

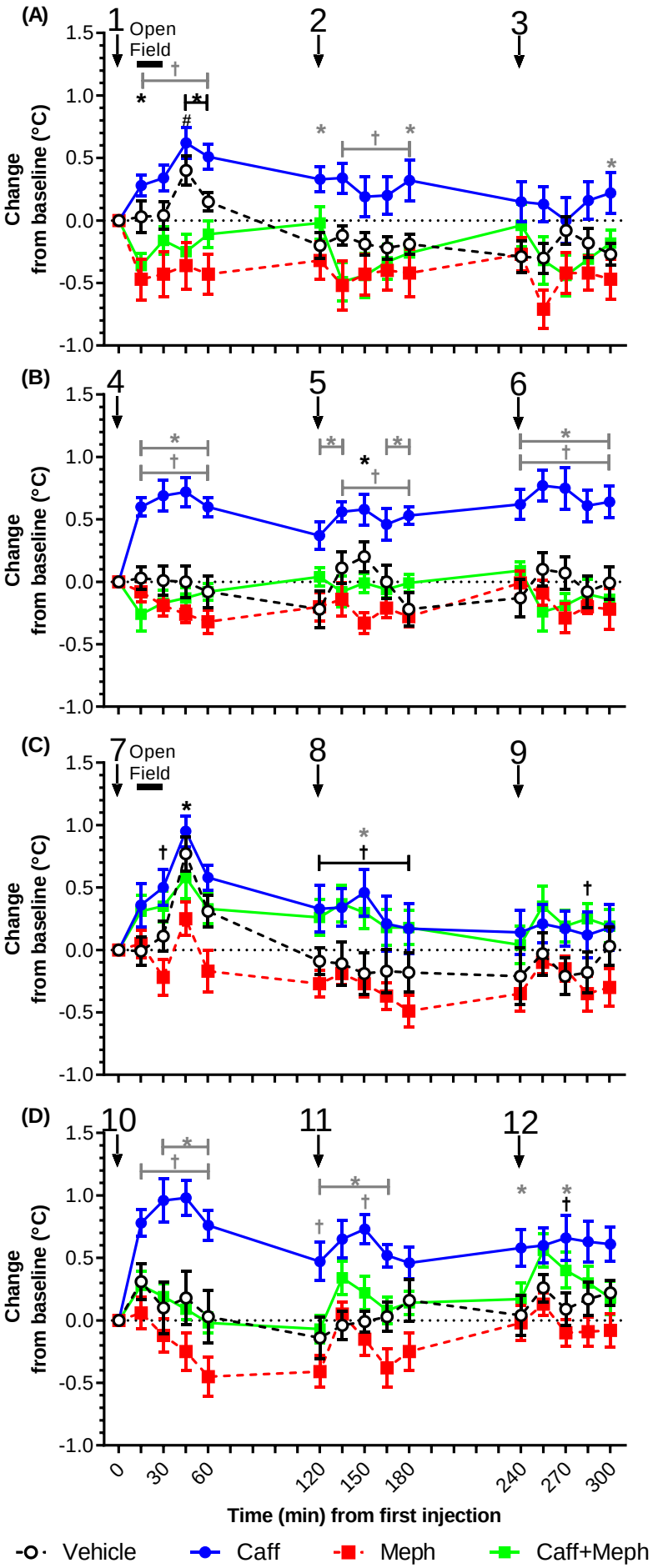
Following drug injections there was generally a main effect of time, but not on day 2 (statistics for all effects are depicted in **Table 2.2**). There was always a main effect of caffeine, which showed no interaction with time. However, post-hoc tests revealed caffeine-induced hyperthermia was evident at isolated time-points following the first injection of each week, and increased in magnitude and duration on the second day of each week **(Fig 2.11)**.

There were main effects of mephedrone on three of the four injection days, but not day 8, and always a mephedrone x time interaction. Mephedrone-induced hypothermia was evident following at least one injection on days 1 **(Fig. 2.11A)**, 2 **(Fig. 2.11B)** and 8 **(Fig. 2.11C)**, although only at isolated time points.

Interestingly, there was a mephedrone x caffeine interaction on day 2 – but on no other day – when caffeine attenuated mephedrone-induced hypothermia, although at no isolated time point did this reach statistical significance. Of greater importance, on day 8 a significant increase in body temperature in combination-, relative to mephedrone-treated rats, was observed following all injections **(Fig. 2.11C)**, whereby caffeine converted mephedrone-induced hypothermia to mild hyperthermia. This was also observed following the third injection on day 9 **(Fig. 2.11D)**, although the only case of a significant elevation in body temperature, relative to vehicle-treated rats, was observed following the first injection on day 1 **(Fig. 2.11A)**. However, no mephedrone x caffeine x time interaction effect was observed at any point.

Table 2.2 Main and interaction effects of time, caffeine and mephedrone on body temperature. Significant effects are depicted in white boxes, whilst non-significant effects are depicted in grey boxes.

	Day 1	Day 2	Day 8	Day 9
Time	$F_{(13,\ 468)} = 5.061$, $P < 0.001$	$F_{(13,\ 468)} = 1.257$, $P = 0.236$)	$F_{(13,\ 468)} = 23.878$, $P < 0.001$	$F_{(13,\ 468)} = 7.168$, $P < 0.001$
Caff	$F_{(1,\ 36)} = 6.844$, $P < 0.05$	$F_{(1,\ 36)} = 19.796$, $P < .001$	$F_{(1,\ 36)} = 10.660$, $P < 0.01$	$F_{(1,\ 36)} = 18.142$, $P < 0.001$
Meph	$F_{(1,\ 36)} = 17.631$, $P < 0.001$	$F_{(1,\ 36)} = 29.445$, $P < 0.001$	$F_{(1,\ 36)} = 1.017$, $P = 0.320$	$F_{(1,\ 36)} = 10.863$, $P < 0.01$
Time x Caff	$F_{(13,\ 468)} = 1.291$, $P = 0.214$	$F_{(13,\ 468)} = 1.220$, $P = 0.261$	$F_{(13,\ 468)} = 1.600$, $P = 0.082$	$F_{(13,\ 468)} = 1.223$, $P = 0.259$
Time x Meph	$F_{(13,\ 468)} = 3.489$, $P < 0.001$	$F_{(13,\ 468)} = 3.421$, $P < 0.001$	$F_{(13,\ 468)} = 3.482$, $P < 0.001$	$F_{(13,\ 468)} = 3.762$, $P < 0.001$
Caff x Meph	$F_{(1,\ 36)} = 0.970$, $P = 0.331$	$F_{(1,\ 36)} = 9.973$, $P < 0.01$	$F_{(1,\ 36)} = 0.291$, $P = 0.593$	$F_{(1,\ 36)} = 1.026$, $P = 0.318$
Time x Caff x Meph	$F_{(13,\ 468)} = 0.492$, $P = 0.929$	$F_{(13,\ 468)} = 1.581$, $P = 0.087$	$F_{(13,\ 468)} = 0.880$, $P = 0.574$	$F_{(13,\ 468)} = 0.214$, $P = 0.173$


(A)
(B)
(C)
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1
2
3
4
5
6
7
8
9
10
11
12
Open Field
Change from baseline (°C)
1.5
1.0
0.5
0.0
-0.5
-1.0
0
30
60
120
150
180
240
270
300
Time (min) from first injection
Vehicle
Caff
Meph
Caff+Meph

Figure 2.12 Effect of repeated administration of mephedrone, with and without caffeine, on subcutaneous body temperature. Group-housed adolescent male Lister hooded rats (n = 10 per treatment group; PND 34/35 at the time of first injection) received 12 separate i.p. injections of saline vehicle (saline, 1.0 ml kg^{-1}), mephedrone (7.5 mg kg^{-1}; Meph), caffeine (10.0 mg kg^{-1}; Caff) or mephedrone and caffeine (combined in the same injection; Caff + Meph) thrice daily (2 h apart) on two consecutive days for two weeks (as indicated by numbered arrows). Subcutaneous temperature was measured via a previously implanted (approx. –7 days) microchip immediately prior to each injection and at 15 min intervals 60 min thereafter. Data are presented as temperature change (mean ± SEM) from the baseline (0 min) reading on days 1 **(A)**, 2 **(B)**, 8 **(C)** and 9 **(D)**. All statistical analyses refer to: $^{*}P < 0.05$ Caff, $^{**}P < 0.05$ Meph and $^{\#}P < 0.05$ Caff + Meph versus Vehicle; $^{\dagger}P < 0.05$ Caff and $^{\dagger}P < 0.05$ Meph versus Caff + Meph. Absolute baseline data (i.e. at time zero) were excluded from statistical analyses. Data for each day were analysed using Tukey's multiple comparisons post-hoc test following three-way repeated measures ANOVA.

2.4.1.3 Behaviour in the home cage

The behaviour of rats in the home cage was observed 15 min following each injection. There was no significant effect of injection number on any day (statistics for all effects are depicted in **Table 2.3**).

There was always a main effect of caffeine, which interacted with injection number on day 1, but at no other point. However, post-hoc tests revealed caffeine caused a significant increase in home cage behavioural score on all days. Caffeine produced comparatively greater increases in home cage behavioural scores following successive injections on day 1.

There was also always a main effect of mephedrone, which interacted with injection number on all days, with the exception of day 1. Post-hoc analyses revealed mephedrone caused a significant increase in behavioural rating scores of rats on all days, and these scores were comparatively greater following successive injections.

No significant mephedrone x caffeine interaction effect was observed at any point during the experiment, nor was a further interaction observed with injection number. However, post-hoc tests revealed that co-administration of caffeine and mephedrone caused increases in behavioural scores, relative to vehicle-treated animals, following the second and third injection of each day; and relative to mephedrone-treated animals on days 1 **(Fig. 2.12A)** and 8 **(Fig. 2.12C)**.

Table 2.13 Main and interaction effects of injection number (Inj. no.), caffeine (Caff) and mephedrone (Meph) on behavioural observation scores. Significant effects are depicted in white boxes, whilst non-significant effects are depicted in grey boxes.

	Day 1	Day 2	Day 8	Day 9
Inj. no.	$F_{(2, 72)} = 0.023$, $P = 0.978$	$F_{(2, 72)} = 1.981$, $P = 0.145$	$F_{(2, 72)} = 0.949$, $P = 0.392$	$F_{(2, 72)} = 0.146$, $P = 0.865$
Caff	$F_{(1, 36)} = 19.510$, $P < 0.001$	$F_{(1, 36)} = 12.709$, $P < 0.001$	$F_{(1, 36)} = 22.372$, $P < 0.001$	$F_{(1, 36)} = 7.346$, $P < 0.05$
Meph	$F_{(1, 36)} = 28.630$, $P < 0.001$	$F_{(1, 36)} = 12.709$, $P < 0.001$	$F_{(1, 36)} = 32.664$, $P < 0.001$	$F_{(1, 36)} = 16.168$, $P < 0.001$
Inj. no. x Caff	$F_{(2, 72)} = 3.425$, $P < 0.05$	$F_{(2, 72)} = 0.494$, $P = 0.612$	$F_{(2, 72)} = 2.765$, $P = 0.070$	$F_{(2, 72)} = 2.714$, $P = 0.073$
Inj. no. x Meph	$F_{(2, 72)} = 2.765$, $P = 0.070$	$F_{(2, 72)} = 3.804$, $P < 0.05$	$F_{(2, 72)} = 4.141$, $P < 0.05$	$F_{(2, 72)} = 4.517$, $P < 0.05$
Caff x Meph	$F_{(1, 36)} = 1.580$, $P = 0.217$	$F_{(1, 36)} = 0.432$, $P = 0.515$	$F_{(1, 36)} = 0.621$, $P = 0.436$	$F_{(1, 36)} = 0.200$, $P = 0.658$
Inj. no. x Caff x Meph	$F_{(2, 72)} = 1.232$, $P = 0.298$	$F_{(2, 72)} = 0.393$, $P = 0.670$	$F_{(2, 72)} = 0.281$, $P = 0.756$	$F_{(2, 72)} = 0.116$, $P = 0.891$

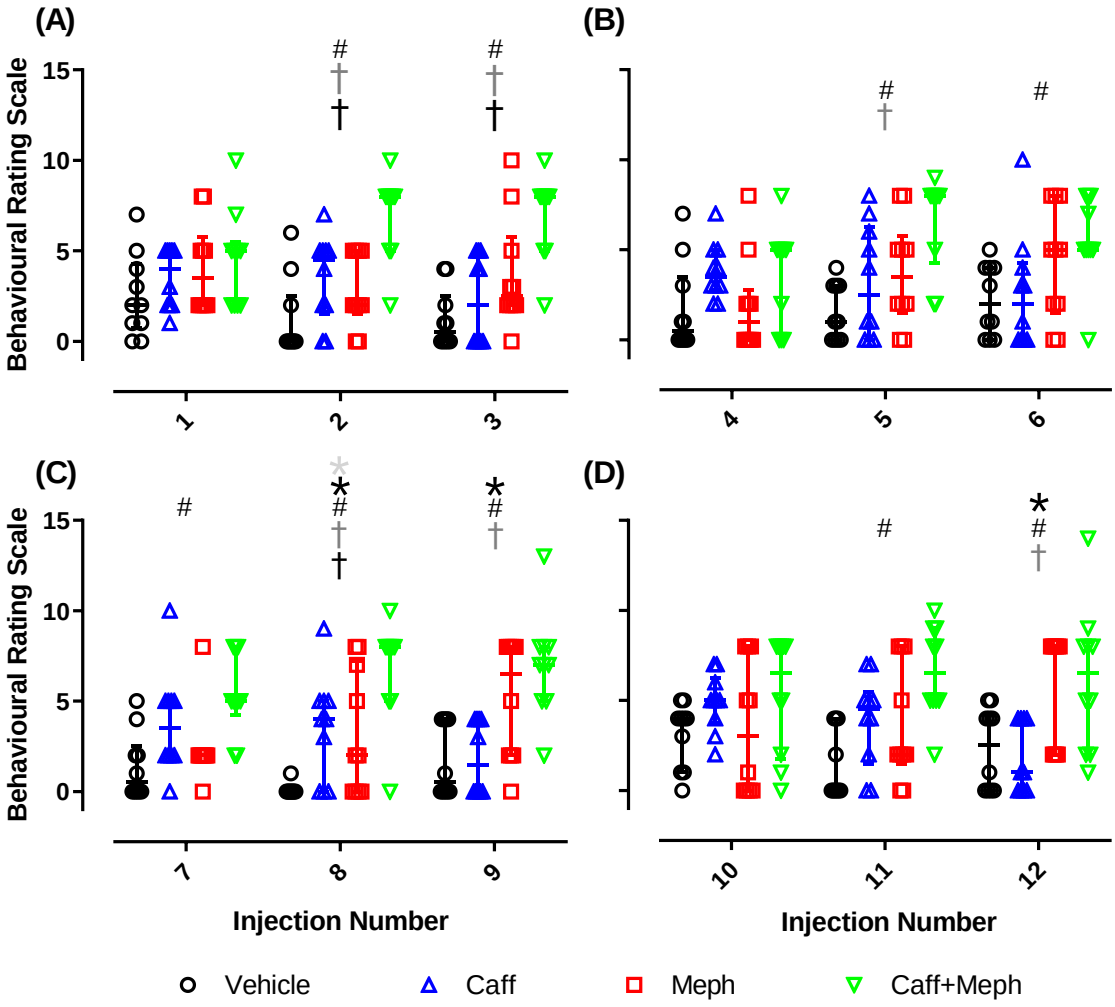

Figure 2.14 Effect of repeated administration of mephedrone, with and without caffeine, on home cage behaviour. Group-housed adolescent male Lister hooded rats (n = 10 per treatment group) received i.p. saline vehicle (1.0 ml kg^{-1}), mephedrone (7.5 mg kg^{-1}; Meph), caffeine (10.0 mg kg^{-1}; Caff) or mephedrone and caffeine (combined in the same injection; Caff + Meph) thrice daily (2 h apart), with home cage behaviour observed 15 min post-injection on days 1 **(A)**, 2 **(B)**, 8 **(C)** and 9 **(D)**. Data are presented as behavioural rating scale score (median with interquartile range), with no animals consistently appearing as an outlier throughout all observations. All statistical analyses refer to: *$P < 0.05$ Caff, ***$P < 0.05$ Meph and #$P < 0.05$ Caff + Meph versus Vehicle; †$P < 0.05$ Caff and ✝$P < 0.05$ Meph versus Caff + Meph. Data for each day were analysed using Tukey's multiple comparisons post-hoc test following two-way repeated measures ANOVA.

2.4.1.4 Open field

Open field behaviour, including distance moved and time spent in the central zone, was measured 30 min following the first injection of each week, for 15 min. Selection of this time window was predicated on the timing of peak effects of mephedrone administration observed in human users (Papaseit *et al.*, 2016). On both days, there was a main effect of caffeine on time spent in the central zone (day 1: $F_{(1,36)} = 12.486$, $P < 0.01$; day 8: $F_{(1, 36)} = 8.835$, $P < 0.01$). However, a main effect of caffeine on distance moved was observed only on day 8 ($F_{(1,36)} = 10.897$, $P < 0.01$), and not day 1 ($F_{(1,36)} = 2.761$, $P = 0.105$). Likewise, no main effect of caffeine was observed on faecal pellet expulsion (day 1: $F_{(1, 36)} = 0.005$, $P = 0.941$; day 8: $F_{(1, 36)} = 3.049$, $P = 0.089$), grooming (day 1: $F_{(1, 36)} = 0.419$, $P = 0.522$; day 8: $F_{(1, 36)} = 0.539$, $P = 0.468$) or unsupported rearing in the central zone (day 1: $F_{(1, 36)} = 0.274$, $P = 0.604$; day 8: $F_{(1, 36)} = 1.334$, $P = 0.256$). Post-hoc tests revealed that, relative to vehicle-treated rats, caffeine increased time spent in the central zone on both days, and increased the total distance moved on day 8.

A main effect of mephedrone on distance moved was observed on day 1 ($F_{(1 ,36)} = 8.850$, $P < 0.01$), but not on day 8 ($F_{(1,36)} = 0.010$, $P = 0.920$). Similarly, a main effect was observed on faecal pellet expulsion on day 1 ($F_{(1, 36)} = 12.086$, $P < 0.01$) but not day 8 ($F_{(1,36)} = 0.536$, $P = 0.469$), nor on either day on time spent in the central zone (day 1: $F_{(1,36)} = 0.382$, $P = 0.541$; day 8: $F_{(1,36)} = 0.073$, $P = 0.789$), grooming ($F_{(1, 36)} = 1.076$, $P = 0.306$; day 8: $F_{(1, 36)} = 0.616$, $P = 0.438$), or unsupported rearing in the central zone (day 1: $F_{(1, 36)} = 0.284$, $P = 0.598$; day 8: $F_{(1, 36)} = 0.151$, $P = 0.700$). Post-hoc tests revealed mephedrone increased distance moved relative to vehicle-treated rats, on day 1.

A significant interaction effect between caffeine and mephedrone, on distance moved, was observed on day 8 ($F_{(1, 36)} = 8.516$, $P < 0.01$), but not day 1 ($F_{(1, 36)} = 0.746$, $P = 0.394$). No such interaction effect was observed on time spent in the central zone (day 1: $F_{(1, 36)} = 0.437$, $P = 0.513$; day 8: $F_{(1, 36)} = 1.194$, $P = 0.282$), faecal pellet expulsion (day 1:

$F_{(1, 36)}$ = 0.049, P = 0.826; day 8: $F_{(1, 36)}$ = 0.003, P = 0.955), grooming (day 1: $F_{(1, 36)}$ = 3.798, P = 0.059; day 8: $F_{(1, 36)}$ = 0.179, P = 0.675), or unsupported rearing in the central zone (day 1: $F_{(1, 36)}$ = 0.231, P = 0.634; day 8: $F_{(1, 36)}$ = 0.239, P = 0.628). Post-hoc tests revealed that the co-administration of caffeine and mephedrone elicited an increase in distance moved on day 1 relative to vehicle-treated controls, whilst on day 8, the co-administration of mephedrone prevented caffeine-induced increases in distance moved **(Fig. 2.13A)**, and that, relative to mephedrone-treated rats, combination treatment caused an increase in time spent in the central zone **(Fig. 2.13B)**.

There was a main effect of week on distance moved ($F_{(1,36)}$ = 6.651, P < 0.05) and time spent in the central zone ($F_{(1,36)}$ = 7.636, P < 0.01). Post-hoc analyses revealed that mephedrone elicited an increase in distance moved on day 1, but not on day 8, relative to vehicle-treated animals, suggesting the development of tolerance to the locomotor stimulant effect of mephedrone. Additionally, the co-administration of caffeine with mephedrone elicited an increase in time spent in the central zone, relative to mephedrone-treated animals, on day 8 but not on day 1, indicating that caffeine exhibited a greater capacity for an anxiolytic effect on day 8.

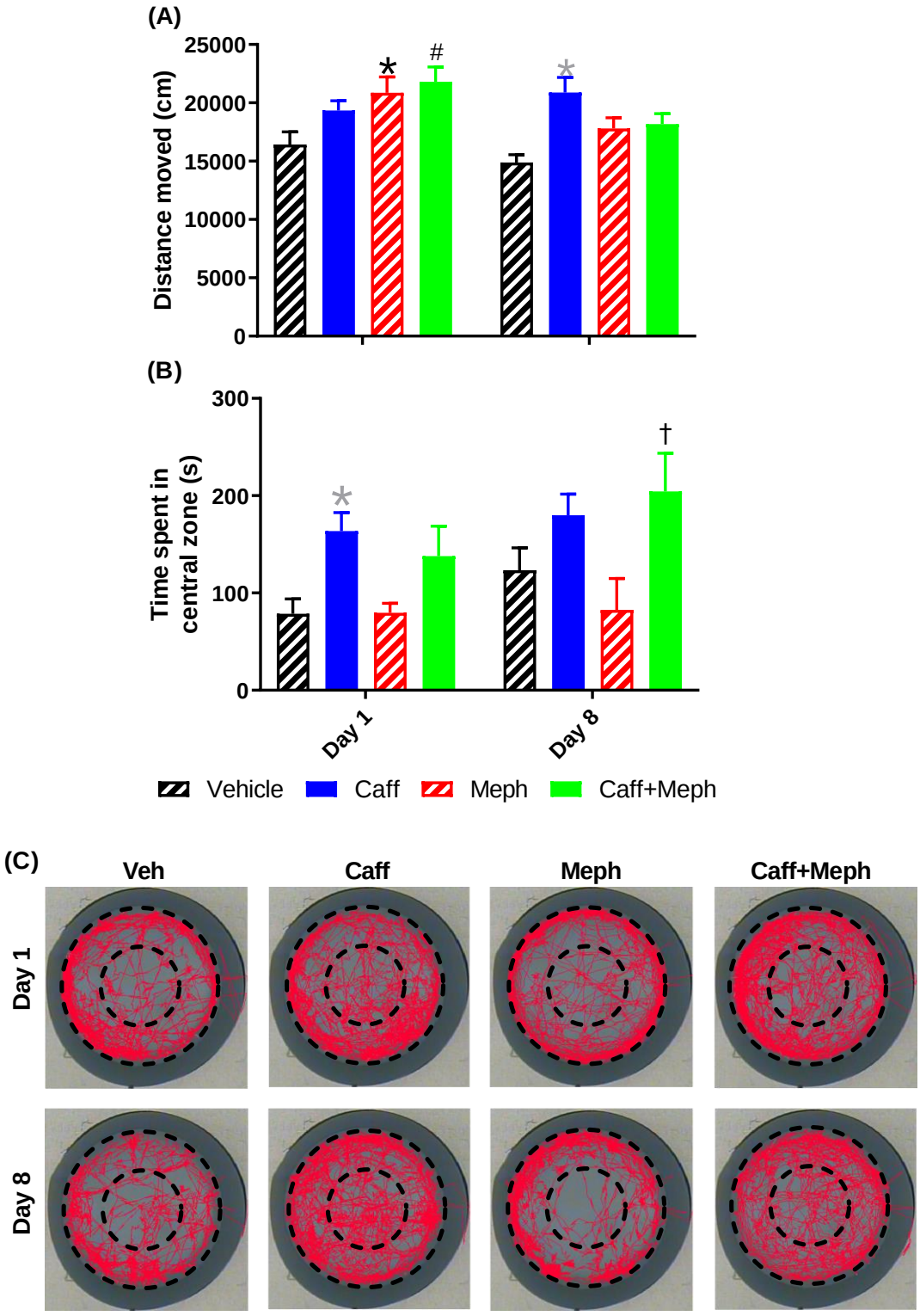

Figure 2.15 Effect of mephedrone, with and without caffeine, on open field test behaviour. Group-housed adolescent male Lister hooded rats (n = 10 per treatment group) received i.p. vehicle (saline, 1.0 ml kg^{-1}), mephedrone (7.5 mg kg^{-1}; Meph), caffeine (10.0 mg kg^{-1}; Caff) or mephedrone and caffeine (combined in the same injection; Caff + Meph) thrice daily (2 h apart), with open field test behaviour assessed

30 min following the first injection of each week (days 1 and 8). Total distance moved **(A)** and duration spent in central zone **(B)** were measured, displayed alongside representative track images (red lines) with computer-defined central and peripheral zones (segmented black lines) **(C)**. Data are presented as distance moved (cm) or time spent in central zone (s) (mean ± SEM). All statistical analyses refer to: *$P < 0.05$ Caff, **$P < 0.05$ Meph and #$P < 0.05$ Caff + Meph versus Vehicle; †$P < 0.05$ Caff and †$P < 0.05$ Meph versus Caff + Meph. Data for each day were analysed using Tukey's multiple comparisons post-hoc test following three-way repeated measures ANOVA (with the exception of data analysed across days, which were analysed with a four-way repeated measures ANOVA).

2.4.1.5 Ultrasonic vocalisations

USVs were measured from 30 min following the first injection of each week for 15 min, whilst rats underwent open field testing. No aversive (~22 kHz) calls were detected by the software, so only positive state-related (~50 kHz) calls were analysed. On both days, there was no main effect of treatment on total number of USVs emitted (day 1: $H_{(3)} = 1.896$, $P = 0.602$; day 8: $H_{(3)} = 6.278$, $P = 0.068$), number of flat calls (day 1: $H_{(3)} = 0.983$, $P = 0.816$; day 8: $H_{(3)} = 6.864$, $P = 0.071$), or the number of step calls (day 1: $H_{(3)} = 3.714$, $P = 0.354$; day 8: $H_{(3)} = 3.664$, $P = 0.416$). There was also no main effect of treatment on the number of trill calls emitted on day 1 ($H_{(3)} = 3.993$, $P = 0.266$), though a significant effect was observed on day 8 ($H_{(3)} = 8.226$, $P < .05$). However, Dunn's multiple comparisons post-hoc revealed no significant differences in the number of trill calls emitted by any treatment group on day 8 **(Fig. 2.14)**. On days 1 and 8, rats emitted, respectively, no more than 12 and 7 USVs in total. However, on day 8, four rats (two treated with saline vehicle, two with caffeine) emitted at least 25 calls each, with one vehicle-treated rat emitting a total of 53 calls.

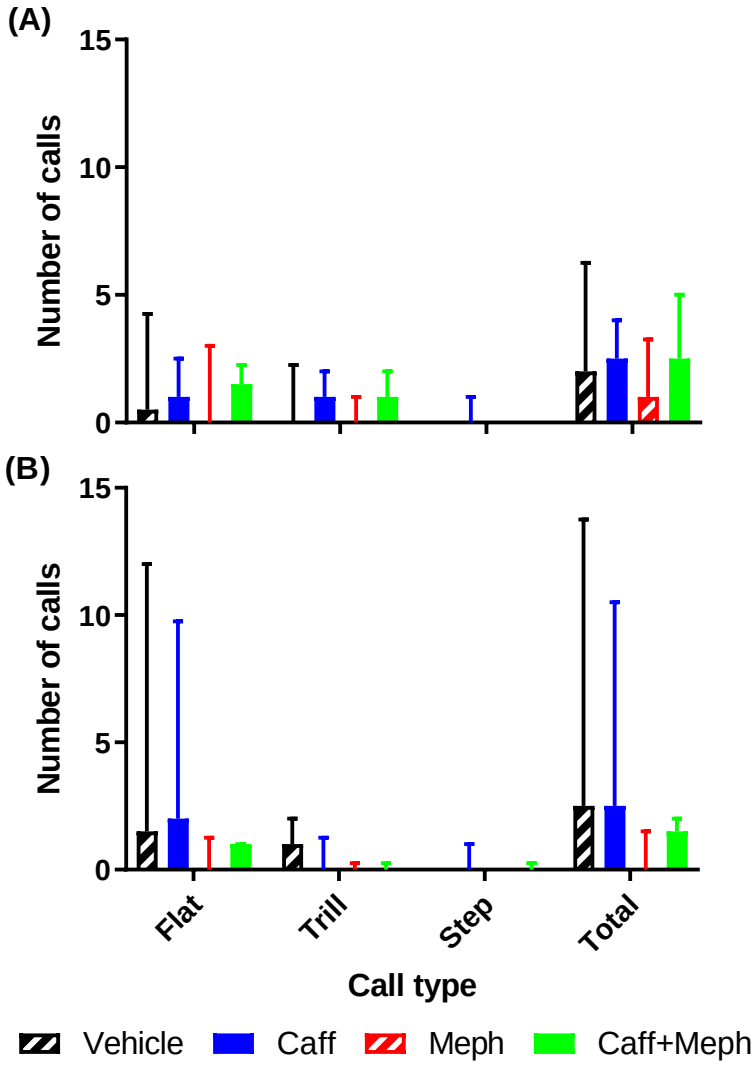

Figure 2.16 Effect of mephedrone, with and without caffeine, on USV emission during the open field test. Group-housed adolescent male Lister hooded rats (n = 10 per treatment group) received i.p. vehicle (saline, 1.0 ml kg^{-1}), mephedrone (7.5 mg kg^{-1}; Meph), caffeine (10.0 mg kg^{-1}; Caff) or mephedrone and caffeine (combined in the same injection; Caff + Meph) thrice daily (2 h apart). Number of USVs and subtypes of USVs were recorded from 30 minutes following administration, for the duration of open field testing on **(A)** day 1 and **(B)** day 8. Data are presented as total number of USVs (median with interquartile range). Data were analysed by Dunn's multiple comparisons post-hoc test following individual Kruskal-Wallis H tests, and are presented as median ± interquartile range.

2.4.1.6 Locomotor activity

When LMA was assessed on PND 59/61/62/63, habituation to activity boxes was confirmed by a decline in ambulation, rearing and fine movement ($P > 0.001$ for all groups, for all three measures) over the 60 min period, with no significant between-group differences **(Fig. 2.15)**. There was no influence of either drug alone or in combination on ambulation (caffeine: $F_{(1, 36)} = 0.209$, $P = 0.650$; mephedrone: $F_{(1, 36)} = 0.992$, $P = 0.326$; caffeine x mephedrone: $F_{(1, 36)} = 1.599$, $P = 0.214$), fine movement (caffeine: $F_{(1, 36)} = 0.672$, $P = 0.418$; mephedrone: $F_{(1, 36)} = 0.165$, $P = 0.687$; caffeine x mephedrone: $F_{(1, 36)} = 2.389$, $P = 0.131$), or rearing (caffeine: $F_{(1, 36)} = 0.002$, $P = 0.965$; mephedrone: $F_{(1, 36)} = 0.331$, $P = 0.569$; caffeine x mephedrone: $F_{(1,36)} = 0.125$, $P = 0.725$).

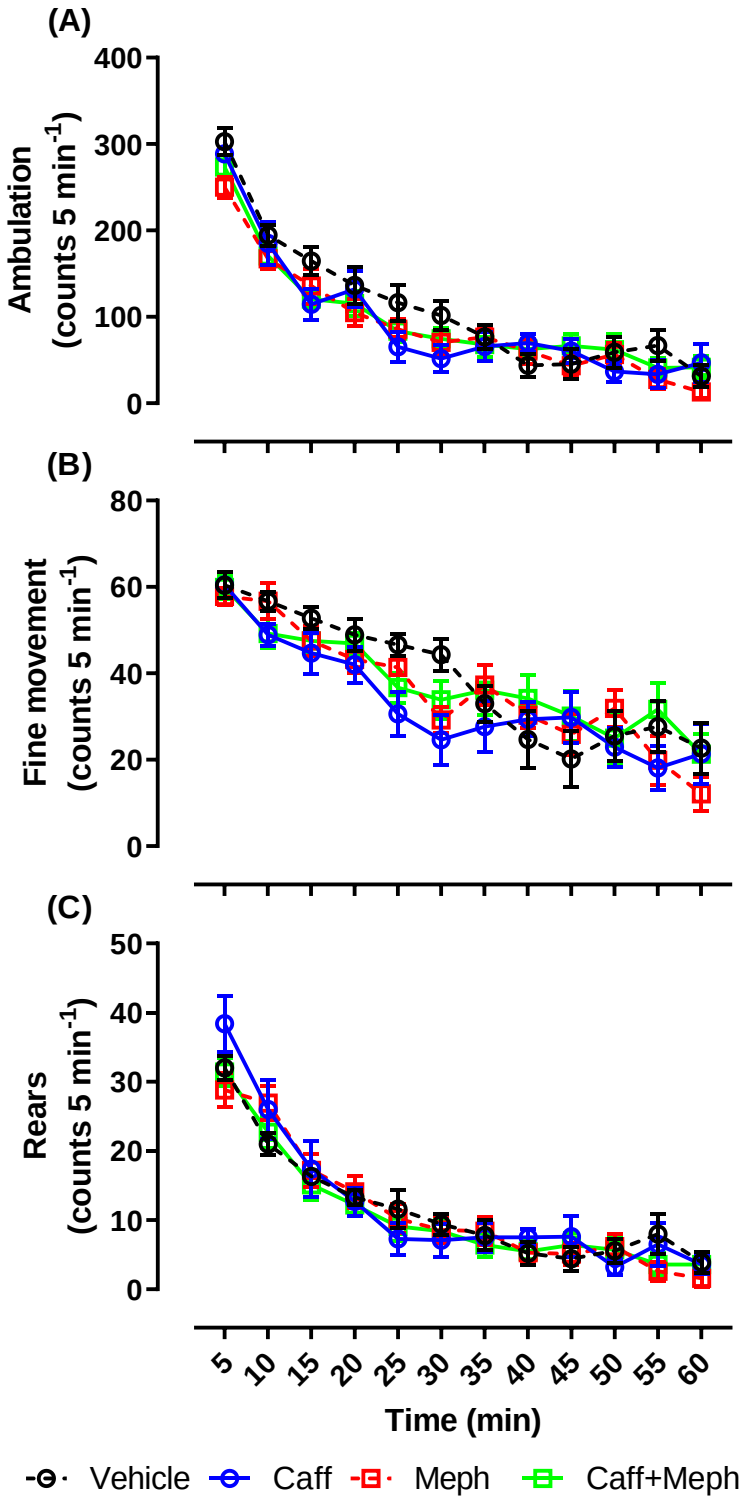

Figure 2.17 All rats displayed habituation to the activity boxes in the 60 min period. Adult male Lister hooded rats (n = 10 per treatment group) displayed habituation to the activity boxes, as shown by decrease in counts of ambulation **(A)**, fine movement **(B)**, and rearing **(C)** (mean ± SEM), when tested on either PND 59, 61, 62 or 63. There

was no significant difference in ambulation, fine movement or rearing between treatment groups.

2.4.1.7 Novel object discrimination

When NOD was assessed on PND 61/63/64, no animals expressed an explorative preference for either of the identical objects in the familiarisation trial ($P > 0.05$, **Fig. 2.16A**). Similarly, no effect of treatment group was noted on total time of object exploration during the familiarisation trial (caffeine: $F_{(1, 36)} = 1.796$, $P = 0.189$; mephedrone: $F_{(1, 36)} = 0.455$, $P = 0.504$; caffeine x mephedrone: $F_{(1, 36)} = 1.015$, $P = 0.320$).

Two hours later, in the choice trial, all groups successfully discriminated the novel from familiar object, irrespective of treatment ($P > 0.001$, **Fig. 2.16B**), with no significant between-groups difference in discrimination ratio ($P > 0.05$, **Fig. 2.16C**). No effect of treatment group was noted on the total time of object exploration during the choice trial (caffeine: $F_{(1, 36)} = 0.532$, $P = 0.471$; mephedrone: $F_{(1,36)} = 0.387$, $P = 0.538$; caffeine x mephedrone: $F_{(1, 36)} = 0.266$, $P = 0.609$; data not shown), indicating no long-term effect of drug treatment on this measure.

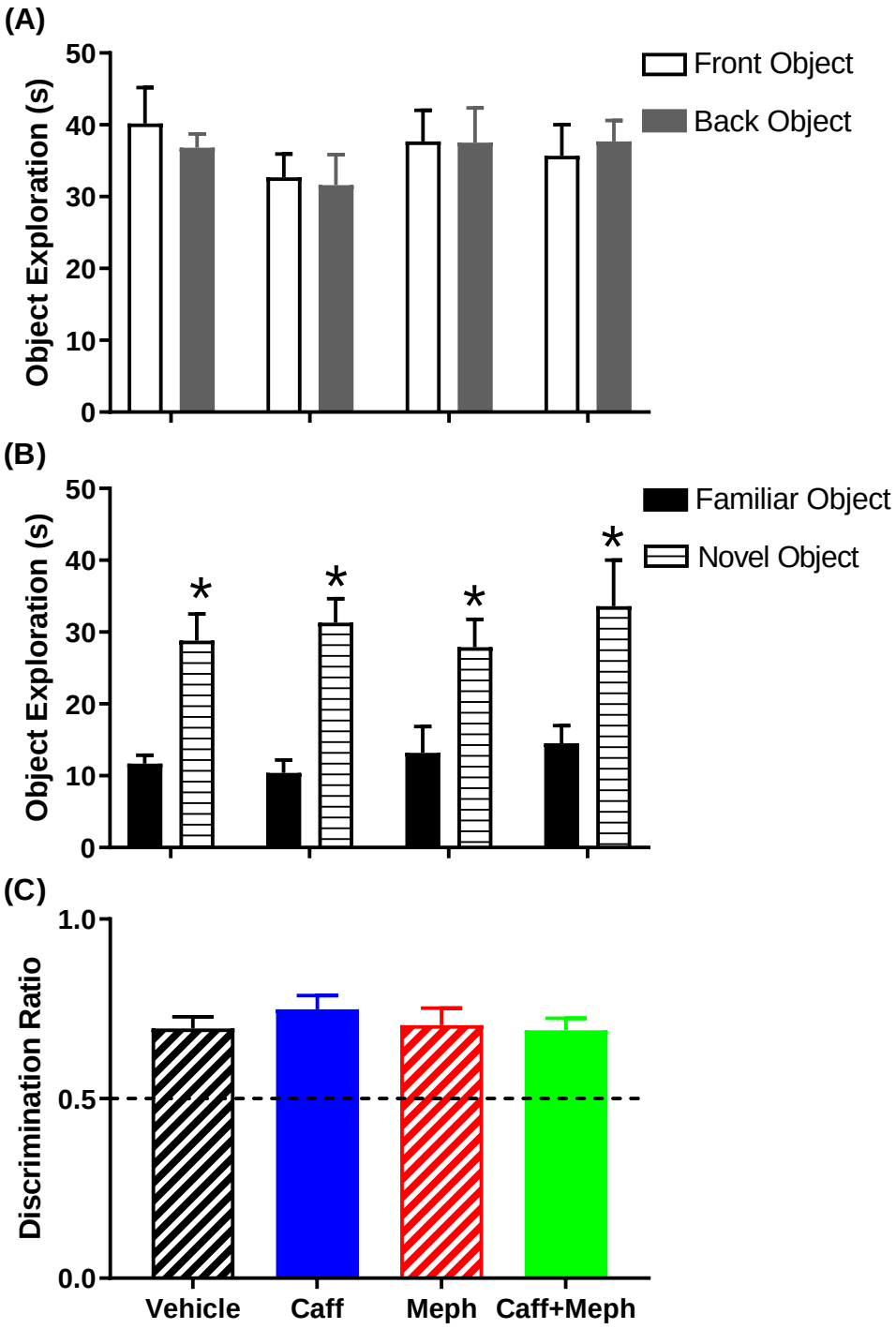

Figure 2.18 Novel object discrimination unimpaired in all treatment groups. Adult male Lister hooded rats (n = 10 per treatment group) were assessed for NOD performance on PND 61/63/64, approximately three weeks following final drug administration. No group exhibited any exploratory preference for either object during the familiarisation trial **(A)**, and all groups successfully discriminated the novel object during the choice trial, as indicated by exploration time **(B)** and discrimination ratio

(C). Data analysed by three-way repeated measures ANOVA, and are presented as mean ± SEM.

2.4.1.8 Elevated plus maze

When EPM was assessed on PND 67/69/70/71, there was no main effect of either treatment group alone, or interaction effect of treatment combination, on distance moved (caffeine: $F_{(1, 35)}$ = 0.143, P = 0.708; mephedrone $F_{(1, 35)}$ = 0.065, P = 0.801; caffeine x mephedrone: $F_{(1, 35)}$ = 1.422, P = 0.241; data not shown), entry frequencies (caffeine: $F_{(1, 35)}$ = 0.265, P = 0.610; mephedrone: $F_{(1, 35)}$ = 0.303, P = 0.586; caffeine x mephedrone: $F_{(1, 35)}$ = 0.126, P = 0.724; data not shown), latency to first entry (caffeine: $F_{(1, 35)}$ = 1.449, P = 0.237; mephedrone: $F_{(1, 35)}$ = 1.278, P = 0.266; caffeine x mephedrone: $F_{(1, 35)}$ = 0.185, P = 0.669; data not shown), number of unprotected head dips (caffeine: $F_{(1, 35)}$ = 0.022, P = 0.883; mephedrone: $F_{(1, 35)}$ = 0.171, P = 0.682; caffeine x mephedrone: $F_{(1, 35)}$ = 0.353, P = 0.556), number of peripheral stretch attends (caffeine: $F_{(1, 35)}$ = 0.920, P = 0.344; mephedrone: $F_{(1, 35)}$ = 0.003, P = 0.955; caffeine x mephedrone: $F_{(1, 35)}$ = 0.867, P = 0.358; data not shown), or percentage time in open arms (caffeine: $F_{(1, 35)}$ = 1.171, P = 0.287; mephedrone: $F_{(1, 35)}$ = 2.310, P = 0.138; caffeine x mephedrone: $F_{(1, 35)}$ = 2.183, P = 0.148) **(Fig. 2.17)**. One mephedrone-treated animal was excluded from analyses due to falling from the apparatus during testing. These data suggest no lasting effect of treatment on anxiety-like behaviour in the current behavioural test.

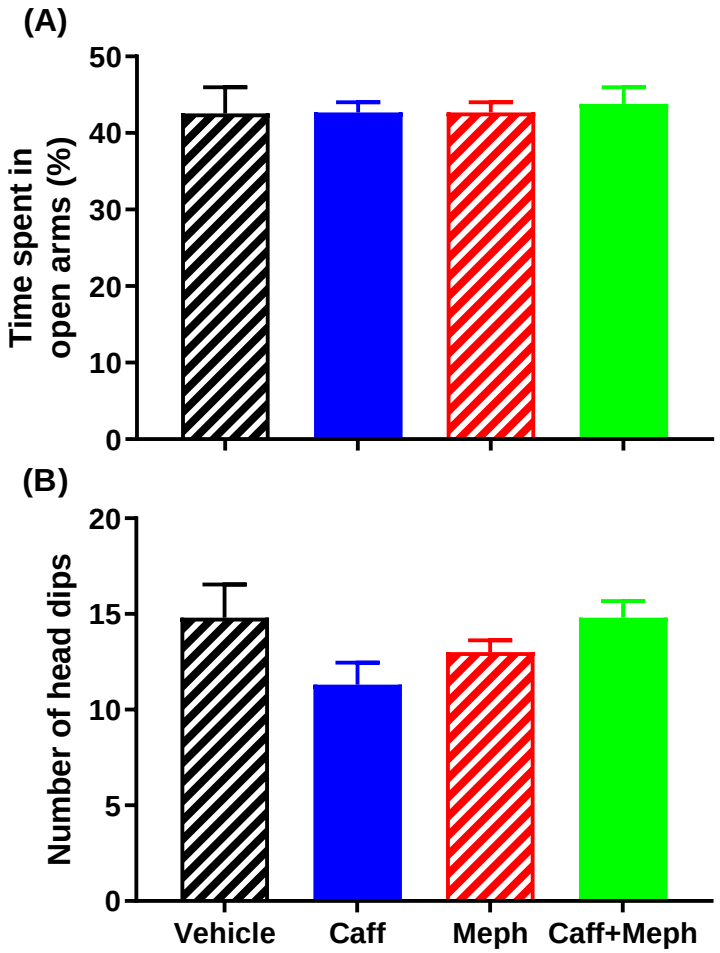

Figure 2.19 "Anxiety-related" behaviours of the elevated plus maze were not affected by any treatment. Adult male Lister hooded rats (n = 9 or 10 per treatment group) were tested on EPM on PND 67/69/70/71, approximately four weeks following final drug administration. There was no effect of treatment on percentage time spent in open arms **(A)**, or unprotected head dips **(B)**. Data analysed by two-way ANOVA, and are presented as mean ± SEM.

2.4.1.9 Pre-pulse inhibition

When PPI was assessed on PND 74/76/77/78, all animals exhibited normal attenuation of the startle exposure to increasing pre-pulse amplitude. There was no main effect of either treatment group alone, or interaction effect of treatment combination, on mean initial startle (caffeine: $F_{(1, 36)} = 0.069$, $P = 0.794$; mephedrone: $F_{(1, 36)} = 0.039$, P

= 0.844; caffeine x mephedrone: $F_{(1, 36)}$ = 0.655, P = 0.424; data not shown), habituation to the startle pulse alone (caffeine: $F_{(1, 36)}$ = 0.068, P = 0.796; mephedrone: $F_{(1, 36)}$ = 0.100, P = 0.754; caffeine x mephedrone: $F_{(1, 36)}$ = 0.166, P = 0.686; data not shown), mean final startle (caffeine: $F_{(1, 36)}$ = 0.053, P = 0.819; mephedrone: $F_{(1, 36)}$ = 0.019, P = 0.891; caffeine x mephedrone: $F_{(1, 36)}$ = 0.001, P = 0.977; data not shown), or % PPI (caffeine: $F_{(1, 36)}$ = 0.001, P = 0.972; mephedrone: $F_{(1, 36)}$ = 0.389, P = 0.537; caffeine x mephedrone: $F_{(1, 36)}$ = 0.057, P = 0.813) **(Fig. 2.18)**, indicating no long-term effect of drug treatment on prepulse inhibition.

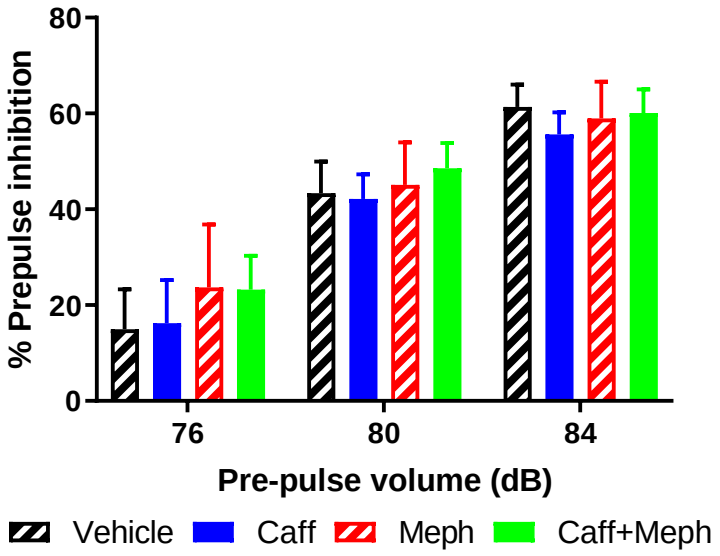

Figure 2.20 Pre-pulse inhibition of the acoustic startle response was not effected by any treatment. Adult male Lister hooded rats (n = 10 per treatment group) were tested for PPI on PND 74/76/77/78, approximately five weeks following final drug administration. There was no effect of treatment on percentage pre-pulse inhibition. Data analysed by three-way repeated measures ANOVA, and are presented as mean ± SEM.

2.4.1.10 Conditioned freezing response

CFR was assessed across two days: acquisition day (PND 80/82/83/84); retention day (PND 81/83/84/85). On acquisition day, there was a main effect of shock number ($F_{(2,}$

$_{72)}$ = 43.80, P < 0.001), with Tukey's multiple comparisons post hoc analyses revealing that rats spent significantly more time freezing in response to the second and third shocks, relative to the first (P < 0.001 in each case). However, there were no main effects of either treatment group alone, or interaction effect of treatment combination, on latency to enter the dark compartment (caffeine: $F_{(1, 36)}$ = 0.642, P = 0.428; mephedrone: $F_{(1, 36)}$ = 0.577, P = 0.452; caffeine x mephedrone: $F_{(1, 36)}$ = 2.126, P = 0.153; data not shown) or freezing duration (caffeine: $F_{(1, 36)}$ = 0.708, P = 0.406; mephedrone: $F_{(1, 36)}$ = 0.899, P = 0.349; caffeine x mephedrone: $F_{(1, 36)}$ = 0.013, P = 0.911) **(Fig. 2.19A)**.

On retention day, a significant main effect of trial number was observed ($F_{(1, 36)}$ = 64.15, P < 0.001), with Šídák's multiple comparisons post hoc analyses revealing freezing time was increased post-cue, relative to pre-cue, for all treatment groups (P < 0.001 for all groups). However, there were no main effects of freezing duration in response to context (caffeine: $F_{(1, 36)}$ = 1.740, P = 0.195; mephedrone: $F_{(1, 36)}$ = 0.010, P = 0.922; caffeine x mephedrone: $F_{(1, 36)}$ = 0.809, P = 0.374) or freezing duration in response to cues (caffeine: $F_{(1, 36)}$ = 0.616, P = 0.438; mephedrone: $F_{(1, 36)}$ = 0.844, P = 0.364; caffeine x mephedrone: $F_{(1, 36)}$ = 0.752, P = 0.392) **(Fig. 2.19B)**. Collectively, these data suggest no lasting effect of drug treatment on conditioned freezing.

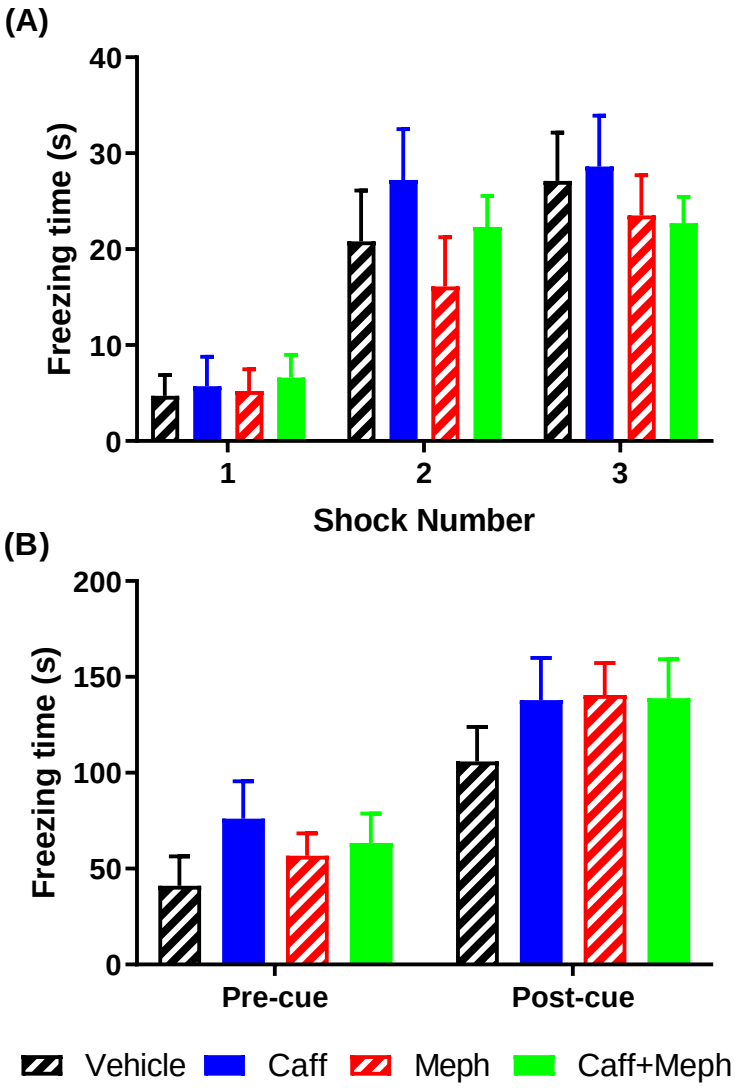

Figure 2.21 Conditioned freezing response was not effected by any treatment. Adult male Lister hooded rats (n = 10 per treatment group) were tested for CFR on PND 81/83/84/85, approximately six weeks following final drug administration. Duration (s, mean ± SEM) of freezing behaviour was measured during acquisition **(A)** and retention **(B)** trials of the CFR task. There was no significant effect on either day. Data analysed by three-way repeated measures ANOVA; two-way ANOVA.

2.4.1.11 Hippocampal microglial activation

Hippocampi were dissected for IHC analyses on PND 82/84/85/86. There was no main effect of either treatment alone, or interaction effect of treatment combination, on Iba-1 positive cell count of CA1 (caffeine: $F_{(1, 34)} = 0.645$, $P = 0.427$; mephedrone: $F_{(1, 34)} = 0.315$, $P = 0.579$; caffeine x mephedrone: $F_{(1, 34)} = 1.227$, $P = 0.276$), dentate gyrus (caffeine: $F_{(1, 34)} = 0.591$, $P = 0.447$; mephedrone: $F_{(1, 34)} = 0.082$, $P = 0.776$; caffeine x mephedrone: $F_{(1, 34)} = 0.025$, $P = 0.875$) or CA3 (caffeine: $F_{(1, 34)} = 1.606$, $P = 0.214$; mephedrone: $F_{(1, 34)} = 1.210$, $P = 0.279$; caffeine x mephedrone: $F_{(1, 34)} = 0.295$, $P = 0.590$) **(Fig. 2.20)**, indicating no lasting effect of drug treatment.

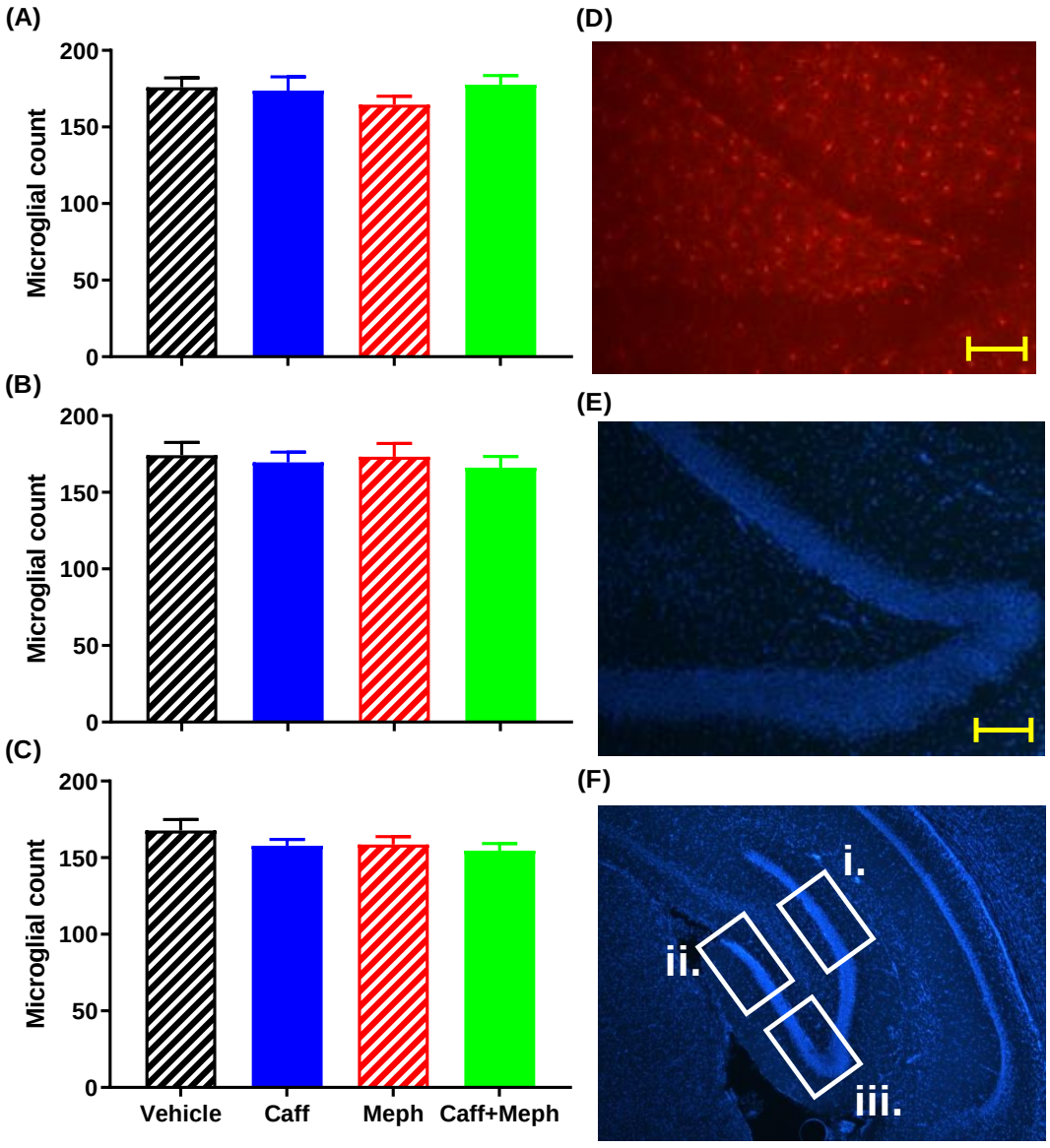

Figure 2.22 Hippocampal Iba-1-positive cell count was not affected by treatment. Adult male Lister hooded rats (n = 10 per treatment group) were killed on PND 82/84/85/86, approximately six weeks following final drug administration. Dorsal hippocampus was dissected and analysed for Iba-1 immunolabelling of the regions CA1 **(A)**, dentate gyrus **(B)** and CA3 **(C)**. Representative images of Iba-1-positive cells **(D)** and nuclei (DAPI) **(E)** in the dentate gyrus (magnification x 20; scale bar equivalent to 500 µM). Between one and five slices were obtained from each rat, with the exception of two (1 x caffeine group; 1 x mephedrone group) for analysis. Approximations of areas magnified (from x 4 to x 20) for subfield analysis of CA1 [i.], CA3 [ii.] and dentate gyrus [iii.] **(F)**. Data are expressed as the number of Iba-1-positive cells stained. No effect of treatment was noted on any region. Data analysed by two-way ANOVA, and are presented as mean ± SEM.

2.5 Discussion

2.5.1 Short-term effect of repeated administration of mephedrone, with and without caffeine, on body temperature and behaviour.

Recreational users of mephedrone report experiencing unpleasant changes in temperature (Dargan *et al.*, 2011; Vardakou *et al.*, 2011) including hot flushes and sweating (Schifano *et al.*, 2011) suggestive of an alteration in peripheral thermoregulation via changes in blood flow. However, unlike for MDMA, hyperthermic reactions are seldom reported in relation to mephedrone (Dargan *et al.*, 2011; Wood *et al.*, 2011), indicating a difference in effect on body temperature which is reflected in the preclinical literature. In rodents, mephedrone's augmentation of body temperature is contingent on dosing regimen and context. Administration at acute and low doses (Aarde *et al.*, 2013; Shortall *et al.*, 2013a; Lopez-Arnau *et al.*, 2015; Shortall *et al.*, 2016b) renders a hypothermic response which is indicatively mediated by 5-HT, as suggested by its prevention by selective 5-HT lesioning with 5,7-DHT or 5-

HT_{1A} antagonism with WAY-100,635 (Shortall *et al.*, 2016b), coupled with the low affinity of mephedrone for the 5-HT_{1A} receptor (Ki > 20μM) (Eshleman *et al.*, 2013; Simmler *et al.*, 2013), and the known action of mephedrone to elevate synaptic 5-HT content through stimulation of release (Kehr *et al.*, 2011; Baumann *et al.*, 2012; Golembiowska *et al.*, 2016) and prevention of reuptake via transporter blockade (Hadlock *et al.*, 2011; Baumann *et al.*, 2012). In contrast, mephedrone-induced hypothermia is abolished upon repeated administration (Shortall *et al.*, 2016b), and is often converted to hyperthermia (Hadlock *et al.*, 2011; Angoa-Perez *et al.*, 2012; Baumann *et al.*, 2012; Martinez-Clemente *et al.*, 2014; Anneken *et al.*, 2017a) – an effect also seen following single administration at high doses (den Hollander *et al.*, 2014a; Grecco & Sprague, 2016; Zona *et al.*, 2016). Prevention of this hyperthermia by subsequent administration of carvedilol (30 min post-drug) suggests mephedrone induces hyperthermia by enhanced activity of α_1- and β-adrenoceptors (Zona *et al.*, 2016). Complementing this, our group previously found the hypothermic response to mephedrone was prolonged by the α_1-adrenoceptor antagonist, prazosin (Shortall *et al.*, 2013a). Overall, mephedrone induced a hypothermia which was attenuated by co-administration of caffeine in the first week, and converted to a mild hyperthermia by caffeine in the second week. The present discrepancies (i.e. the more subtle effect on temperature, relative to previous findings of our group) may be attributable to an additive effect of stress induced by the rectal probe employed for temperature measurement in the previous study described, as opposed to the implanted telemetry devices used herein, or in the lesser dose of mephedrone administered.

In the current study, mephedrone produced significant increases in locomotor activity 30 to 45 min following administration, consistent with previous data of its stimulant effect in rodents within this time frame (Wright *et al.*, 2012; Shortall *et al.*, 2013b; Green *et al.*, 2014; Pail *et al.*, 2015; Nguyen *et al.*, 2016; Shortall *et al.*, 2016a). Previous findings of this group and others indicate these increases are particularly dependent on 5-HT mechanisms, as indicated by their attenuation following blockade of the 5-HT_{2A} receptor with ketanserin (Lopez-Arnau *et al.*, 2012), selective depletion of 5-HT content with 5,7-DHT, or 5-HT_{1B} antagonism with GR 127935 (Shortall *et al.*, 2016b).

Caffeine, a psychostimulant of the xanthine class which transiently improves psychomotor speed in humans (Glade, 2010), acutely increased locomotor activity in the second week of the current study, consistent with previous findings of our group (Shortall *et al.*, 2016a). This was likely mediated by adenosine – particularly A_{2A} – receptor antagonism (Kim & Palmiter, 2003; Chen *et al.*, 2010; Taura *et al.*, 2018). A_{2A} receptors are abundant in the striatum (Alexander & Reddington, 1989; Rosin *et al.*, 1998; Svenningsson *et al.*, 1999), where they are co-localised with dopamine D_2 receptors on striatopallidal GABA neurons (Svenningsson *et al.*, 1999), with which they likely interacted to effect the present increases in locomotor activity.

In a previous study from our group, the co-administration of mephedrone (10.0 mg kg^{-1}) and caffeine elicited an enhancement of the locomotor response relative to caffeine alone (Shortall *et al.*, 2016a). Although such an enhancement was not observed in the current study (and in fact mephedrone appeared to prevent caffeine-induced increases on day eight), this may be attributable to the previous studies differences in testing apparatus (an activity box as described in *2.3.3.5.*), the timing of measurement (immediately following injection for 60 min), or difference in mephedrone dose. It could be the case that the locomotor response to mephedrone observed on day eight is less than on day one because the animals were comparatively less stressed about the procedure, having been acclimatised to handling and the open field apparatus prior to the second exposure.

Anxiety is reported by mephedrone users (Schifano *et al.*, 2011) as the most common of all adverse effects (Carhart-Harris *et al.*, 2011), and is indicatively more common than in MDMA users (Jones *et al.*, 2016). The anxiogenic effect of mephedrone has been replicated in both mouse (0.25 – 10.0 mg kg^{-1}) (Budzynska *et al.*, 2015) and rat (10.0 mg kg^{-1}) (Shortall *et al.*, 2016a), though an anxiolytic effect has been observed in mice following administration at a higher dose (30.0 mg kg^{-1}) (Pail *et al.*, 2015). An online survey of recreational drug users indicates the negation or offset of drug-induced adverse effects is sought via co-administration of another drug or foodstuff

(Sande, 2016). At low doses, caffeine improves mood and reduces anxiety in humans (Quinlan *et al.*, 2000; Lieberman *et al.*, 2002; Haskell *et al.*, 2005), and prevents mephedrone-induced anxiogenic behaviour on the elevated plus maze (Shortall *et al.*, 2016a). Consistent with this previous finding from our group, caffeine produced an anxiolytic effect in the current study, characterised by an increase in time spent in the more aversive central zone of the OFT, complementing the previous suggestion of Shortall *et al.* that caffeine may be co-administered by cognizant recreational drug users in order to ameliorate the effect of anxiety elicited by mephedrone. This anxiolytic effect was not reflected in the USV data, which showed that caffeine did not elicit a significant change in the emission of positive-state related USVs, although no such effect was observed in response to mephedrone, alone or with caffeine.

The development of tolerance towards a drug's effects can be indicative of its abuse liability (although drug abuse can occur in the absence of tolerance) and can compel recreational users, such as those of MDMA/Ecstasy (Verheyden *et al.*, 2003; Parrott, 2005) to increase dosing in an attempt to achieve previously-attained "highs". Tolerance has been reported to develop towards the effects of Khat (Nencini *et al.*, 1984; Wabe, 2011) and synthetic cathinones (Zawilska *et al.*, 2013), including MDPV (Andrabi *et al.*, 2015) and mephedrone (Winstock *et al.*, 2011a; Rácz *et al.*, 2012; Ribeiro *et al.*, 2012). In rodents, the development of tolerance or sensitisation towards MDMA appears contingent on dosing and regimen, whereby the former has been observed following chronic administration at high doses (Callaway & Geyer, 1992), and the latter following repeated intermittent exposure at low doses (Spanos & Yamamoto, 1989; Dafters, 1995; Kalivas *et al.*, 1998; McCreary *et al.*, 1999; Ramos *et al.*, 2004; 2005). The locomotor response to MDMA (5.0 or 10.0 mg kg^{-1}, i.p.), for instance, was attenuated in Sprague-Dawley rats exposed two weeks after previously being administered the drug (4 x 10.0 mg kg^{-1}, i.p., 2 h apart) (Brennan & Schenk, 2006). In the present study, mephedrone elicited significant increases in locomotor activity on day one which were absent on day eight following administration at the same dose, suggesting the development of tolerance to the locomotor stimulant effect of mephedrone. It has been posited that the development of tolerance might be

contingent on drug-induced depletion of 5-HT, as suggested by the attenuation of MDMA-induced hyperactivity following lesioning via 5,7-DHT (5,7-dihydroxytryptamine) (Kehne *et al.*, 1996a) or fenclonine (para-chlorophenylalanine) (Callaway *et al.*, 1990). Coupled with the observations of tolerance following depletion of brain 5-HT as a result of high dose binge administration of MDMA (Baumann *et al.*, 2008a), as well as the impairment of MDMA-induced 5-HT overflow in striatum as a result of prior exposure to MDMA, alongside demonstrable tolerance to MDMA-induced hyperthermia and 5-HT syndrome (Shankaran & Gudelsky, 1999), these literature suggest that tolerance of mephedrones locomotor effects observed in the present study might be a result of mephedrone-induced depletions of brain 5-HT function or content. Nonetheless, there are no neurochemical data to support this contention for the present study.

Mephedrone's pharmacological action to effect increases in synaptic 5-HT content, and the practice of rapid re-dosing and co-administration of other serotonergic-enhancing drugs, present a clinical issue. The co-administration of numerous drugs which precipitate the increase of synaptic 5-HT content via different mechanisms is often a prerequisite of the serotonin syndrome – a syndrome characterised in humans by agitation, tachycardia, diaphoresis, fever, hypertension and clonus amongst other symptoms (Bartlett, 2017), which has previously been observed following co-use of mephedrone and fluoxetine (Garrett & Sweeney, 2010). In preclinical rat models, this syndrome is characterised by numerous stereotyped behaviours, including head weaving and reciprocal forepaw treading (Haberzettl *et al.*, 2013). Caffeine has been evidenced to increase brain 5-HT content *in vitro* and *in vivo* (Nehlig *et al.*, 1992), indirectly through its function as an antagonist at adenosine A_1 and A_2 receptors. Mephedrone, which has previously been shown to elicit reciprocal forepaw treading in rats at a similar dose (10.0 mg kg^{-1}) (Baumann *et al.*, 2012), presently elicited a mild stereotypy with increasing injection number on each day. The co-administration of caffeine precipitated a significant enhancement of this stereotypy, relative to mephedrone-treated animals. It is likely that the differential effects of the two compounds to elicit an increase in synaptic 5-HT content accounts for this.

The effects of cumulative dosing of mephedrone might be in part due to nor-mephedrone, which has been shown to precipitate the release of [^{3}H]-MPP$^+$ and [^{3}H]-5-HT from HEK293 cells and rat brain synaptosomes *in vitro*, as well as to increase accumbal efflux of dopamine and 5-HT, and increase locomotor activity following repeated peripheral administration (Mayer *et al.*, 2016). Of interest, with regard to the observation of mild stereotypy in rats treated only with mephedrone in the present study, Mayer *et al.* also observed nor-mephedrone to elevate accumbal 5-HT efflux in a manner comparable to mephedrone itself. As such, the propensity of both the parent compound and the phase I metabolite to increase synaptic 5-HT content might constitute part of the basis for the induction of the behaviours observed following repeated administration presently.

As in previous studies from this group, the doses of mephedrone and caffeine used in this study caused demonstrable changes in locomotor activity and body temperature, both independently and when co-administered. The generation of stereotyped behaviours characteristic of the serotonin syndrome, particularly in rats repeatedly co-administered mephedrone and caffeine, both warranted and justified further investigation into whether this supposed neurochemical effect precipitated any long-term changes in cognition or microglial activation. The washout period of approximately three weeks was employed to correspond to the transition period of rats from adolescence to adulthood.

2.5.2 Long-term effect of repeated administration of mephedrone, with and without caffeine, on cognition, aversion, and hippocampal microglial activation

Clinical data show significant deficits in semantic-, phonological-, and working-memory in mephedrone users in a state of sobriety (Freeman *et al.*, 2012; Herzig *et al.*, 2013), indicating lasting deficits in these capacities following mephedrone use. The

NOD test – originally described in 1988 (Ennaceur & Delacour, 1988) – is based on the innate preference of the rodent for novelty, and assesses both attention and visual recognition memory (Antunes & Biala, 2012). Mephedrone has previously been shown to impair object discrimination in Wistar rats seven weeks following repeated administration at a high dose (30.0 mg kg^{-1}, daily for ten consecutive days) (Motbey *et al.*, 2012b). However, such impairment was not observed following smaller doses (7.5, 15.0 mg kg^{-1}) (Motbey *et al.*, 2012b), or one week following a four day dosing regimen (30.0 mg kg^{-1}, twice daily, 6 h apart) (den Hollander *et al.*, 2014a). Similarly, the dosing regimen employed in this study failed to precipitate any lasting deficits in object discrimination.

Pre-clinically, the EPM (Walf & Frye, 2007) is used in the aim of assessing anxiety in rodents, based on their aversion to open spaces, and thigmotaxic tendency. Repeated intermittent administration of MDMA has been shown to precipitate an anxiogenic profile in rodents on this task in some studies (Gurtman *et al.*, 2002; Mechan *et al.*, 2002; Faria *et al.*, 2006; Rodriguez-Arias *et al.*, 2011) but not others (Mechan *et al.*, 2002; Bull *et al.*, 2004; Daza-Losada *et al.*, 2008). Mephedrone did not cause long-term changes in anxiety-like behaviour in the current study, consistent with another observation two weeks following repeated high dose administration (30.0 mg kg^{-1}, twice daily, four consecutive days) in C57BL/J6 mice (den Hollander *et al.*, 2013).

PPI is a measure of sensorimotor gating, whereby the presentation of a stimulus inhibits the startle response to a more intense subsequent stimulus (Hoffman & Searle, 1968). Deficiency of PPI is symptomatic of several neuropsychiatric disorders (Geyer *et al.*, 2001), including schizophrenia (Mena *et al.*, 2016). Such deficits have also been observed 39 days following intermittent administration of MDMA (10.0 mg kg^{-1}, s.c., every five days for 15 days, twice per day) to Wistar albino rats during adolescence (Llorente-Berzal *et al.*, 2013), but were not observed as a consequence of repeated administration of mephedrone alone or with caffeine in the present study.

Fear-motivated conditioned freezing is a behaviour observed in numerous animal species – including humans and rodents – which is largely contingent on stimulation of the amygdala. Freezing behaviour is elicited following chemical or electric stimulation of the central nucleus of the amygdala [for review, (Davis & Whalen, 2001)], and can be assessed via the CFR test (Curzon *et al.*, 2009). Previous work by our group found mephedrone to impair hippocampal-dependent associative memory seven days following repeated administration (Shortall *et al.*, 2013b), though no long-term effect on this domain was noted presently.

Microglia are innate immune cells of the central nervous system which function as resident macrophages, mediating immune responses. Ovular in shape, these cells migrate with the use of spindly processes via chemotaxis (Rogers, 2019). Activation of these cells is observed in response to inflammation, such as that observed in several neurodegenerative diseases (Ransohoff & Perry, 2009; Glass *et al.*, 2010), and is thus indicative of neuronal damage. In mice, increased microglial activation has been observed two days following administration of neurotoxic amphetamines, including MDMA (Thomas *et al.*, 2004), though binge administration of mephedrone (4 x 20.0 or 40.0 mg kg^{-1}, i.p. at two hour intervals), failed to produce any lasting changes two or seven days later in striatum (Angoa-Perez *et al.*, 2012). Likewise, administration of a similar regimen (3 x 25.0 mg kg^{-1}, s.c., at two hour intervals, for two days) at high ambient room temperature (26.0 ± 2.0 ^{o}C) was not followed by microglial activation one day following cessation (Lopez-Arnau *et al.*, 2015). Consistent with Angoa-Perez *et al.*, as well as the absence of changes in cognition or aversion, no lasting changes in microglial activation were noted in the hippocampus of rats in the present study.

In sum, these data suggest that repeated administration of mephedrone – at the presently employed dose and regimen – does not precipitate lasting changes in cognition, aversion, or microglial activation of the hippocampus, consistent with a lack of neurotoxic effect observed in previous studies of this group and others. However,

mephedrone has elsewhere been shown to effect oxidative damage following repeated administration at similar doses (Kaminska *et al.*, 2018).

Chapter 3. Probing the role of postsynaptic 5-HT_{1A} receptors in mediating changes in body temperature, locomotor activity and stereotyped behaviour following the combination of mephedrone and caffeine, and measuring neurochemical correlates

3.1 Introduction

In Chapter 2, a single dose of mephedrone was shown to elicit hypothermia and hyperlocomotion which were respectively converted to hyperthermia and enhanced following co-administration of caffeine, whilst this drug combination also elicited mild stereotyped behaviours resemblant of components of the serotonin syndrome. At the time of planning experimental work displayed in the present chapter, existing literature posited that in rats, mephedrone: effects hypothermia via enhanced serotonergic neurotransmission and downstream agonism of 5-HT_{1A} receptors (Shortall *et al.*, 2016b); mediates hyperthermia in part by a direct or indirect agonism of α1- and β-adrenoceptors (Zona *et al.*, 2016); precipitates locomotor hyperactivity chiefly via 5-HT mechanisms, including the 5-HT_{1B} (Shortall *et al.*, 2016b) and 5-HT_{2A} receptors (Lopez-Arnau *et al.*, 2012); and elicits reciprocal forepaw treading, indicative of the serotonin syndrome (Baumann *et al.*, 2012). However, at the time of planning, to the best of the authors knowledge there existed no experimental work probing the pharmacological mechanism(s) by which the combination of mephedrone with caffeine effected changes such as those described in Chapter 2: chiefly, the mild serotonin syndrome observed following combination treatment. In an effort to mitigate this deficit, an *in vivo* microdialysis study was designed, and is described herein.

Commonly reported adverse effects of mephedrone, including unpleasant changes in body temperature, with hot flushes and sweating (Dargan *et al.*, 2011; Schifano *et al.*, 2011; Vardakou *et al.*, 2011), suggest alterations in peripheral thermoregulation via changes in blood flow. In rodents, mephedrone at acute and low doses induces hypothermia (Aarde *et al.*, 2013; Shortall *et al.*, 2013a; Lopez-Arnau *et al.*, 2015; Shortall *et al.*, 2016b) which is blocked by i.c.v. administration of the serotonergic neurotoxin 5,7-DHT (but not the dopaminergic neurotoxin 6-OHDA), whilst the early phase of this response was blocked by prior blockade of the 5-HT_{1A} receptors with WAY-100,635 (Shortall *et al.*, 2016b). This hypothermia is abolished upon repeated administration (Shortall *et al.*, 2016b) and is often converted to hyperthermia (Hadlock

et al., 2011; Angoa-Perez *et al.*, 2012; Baumann *et al.*, 2012; Martinez-Clemente *et al.*, 2014; Anneken *et al.*, 2017a). Hyperthermia is also noted following administration at high doses (den Hollander *et al.*, 2014a; Grecco & Sprague, 2016; Zona *et al.*, 2016), and appears to be mediated in part by activation of α1- and β-adrenoceptors (Zona *et al.*, 2016). It is an effect of potential concern for recreational users, given its association with life-threatening effects of MDMA (Docherty & Green, 2010). In both Chapter 2 and previous observations of this group (Shortall *et al.*, 2016a), mephedrone-induced hypothermia was either abolished or converted to hyperthermia following co-administration of caffeine, and it was suggested that elevation of synaptic 5-HT content is a key component in this effect. A role in mediating this hyperthermia is also suggested for 5-HT_{2A} receptors. This suggestion derives from studies in which hyperthermia, following administration of either the 5-HT precursor 5-hydroxy-L-tryptophan (Krishnamoorthy *et al.*, 2010) and the phenylisopropylamine hallucinogen 1-(2,5-dimethoxy-4-iodophenyl aminopropane (DOI) (Mazzola-Pomietto *et al.*, 1995) has been blocked by 5-HT_{2A} antagonism. It is noteworthy also that mephedrone exhibits binding affinity for this receptor in the millimolar range (Lopez-Arnau *et al.*, 2012; Eshleman *et al.*, 2013; Simmler *et al.*, 2013), indicating a potential for direct action here, though it is unlikely this would produce a notable physiological change in human users.

In keeping with the profile of a psychostimulant, mephedrone elicits locomotor hyperactivity acutely (Wright *et al.*, 2012; Shortall *et al.*, 2013b; Nguyen *et al.*, 2016; Shortall *et al.*, 2016a; Shortall *et al.*, 2016b). To date, prevention of this hyperactivity has been achieved by neurotoxic lesioning of 5-HT content (Shortall *et al.*, 2016b), as well as selective antagonism of the 5-HT_{2A} (Lopez-Arnau *et al.*, 2012), 5-HT_{1B} (Shortall *et al.*, 2016b) or D_1 receptors (Lisek *et al.*, 2012), indicating a role of these three receptors, as well as of 5-HT, in this response. In Chapter 2 and in previous observations from this group (Shortall *et al.*, 2016a), the co-administration of caffeine, which has been shown to increase locomotor activity in its own right (Nehlig *et al.*, 1992), served to enhance mephedrone-induced locomotor hyperactivity, likely in large

part as a consequence of each drug increasing the bioavailability of 5-HT via differing mechanisms, and increasing the likelihood of 5-HT binding to these receptors.

The serotonin syndrome, of which hyperthermia and hyperlocomotion are components in rats, is an adverse effect which has been observed following consumption of two or more serotonergic drugs (Bartlett, 2017), such as bath salt mixtures (Mugele *et al.*, 2012) or mephedrone and fluoxetine (Garrett & Sweeney, 2010) in man. In rats, the syndrome manifests through the exhibition of stereotyped behaviours such as lateral head weaving (Jacobs & Klemfuss, 1975), which occurs upon administration of mephedrone alone (Baumann *et al.*, 2012) and is enhanced by combination with caffeine (Chapter 2). Caffeine has elsewhere been shown to enhance MDMA-induced 5-HT efflux (Gorska & Golembiowska, 2015) via adenosine receptor antagonism, therefore rendering a greater likelihood of this symptom becoming manifest in recreational users, and constituting a cause for concern. In rats, these stereotyped behaviours strongly correlate with dialysate 5-HT content following MDMA administration (Baumann *et al.*, 2008b), which is also elevated by mephedrone (Kehr *et al.*, 2011; Baumann *et al.*, 2012; Wright *et al.*, 2012; Golembiowska *et al.*, 2016; Mayer *et al.*, 2016; Shortall *et al.*, 2016b; Suyama *et al.*, 2016; Lopez-Arnau *et al.*, 2018). Reading of the literature indicates these behaviours can be prevented via 5-HT_{1A} receptor blockade. For instance, pre-administration of WAY-100,635 has been shown to prevent 8-OH-DPAT-induced reciprocal forepaw treading (Forster *et al.*, 1995; Bardin *et al.*, 2001; Kawano *et al.*, 2015) and lateral head weaving (Sato *et al.*, 2012), whilst the latter behaviour has also been prevented by pindolol (Yamaguchi *et al.*, 1987) and methysergide (Fujii *et al.*, 1991). Although there is evidence for the involvement of other receptors in components of the drug-induced serotonin syndrome, such as the prevention of hyperthermia and stereotyped behaviour in adult rats following 5-HT_{2A} antagonism (Krishnamoorthy *et al.*, 2010), most available preclinical literature on these behaviours indicate the involvement of the 5-HT_{1A} receptor.

Baumann *et al.* reported a strong correlation between MDMA-induced lateral head weaving and dialysate dopamine and 5-HT content observed in striatum (Baumann *et al.*, 2008b). Additionally, reductions in PCP-induced lateral head weaving have been noted following lesioning of the striatum by bilateral electrocoagulation (Nabeshima *et al.*, 1983a), and administration of kainic acid, 6-OHDA or 5,6-DHT (Nabeshima *et al.*, 1983b), whilst striatal lesioning via 6-OHDA and transection have prevented lateral head weaving following amphetamine (Andrews *et al.*, 1982), and pargyline with L-tryptophan (Jacobs & Klemfuss, 1975), respectively. Although correlations have also been observed between drug-induced stereotyped behaviours and 5-HT dialysate content of prefrontal cortex (Baumann *et al.*, 2008b), transections or lesions of this area (Scorza *et al.*, 2008), as well as of the cerebellum (Jacobs & Klemfuss, 1975) and nucleus accumbens (Andrews *et al.*, 1982; Nabeshima *et al.*, 1983a), have failed to prevent drug-induced stereotyped behaviours. Based on this, and the major role the striatum plays in motor activity (Schultz, 2000), extracellular 5-HT and dopamine content from this region was measured by *in vivo* microdialysis, so as to observe any correlation between content levels and behavioural effects.

The present study examined how caffeine co-administration modified the acute effects of mephedrone on body temperature, locomotor activity and lateral head weaving, and used the 5-HT_{1A} receptor antagonist WAY-100,635 to determine the extent to which these effects were mediated by downstream involvement of postsynaptic 5-HT_{1A} receptors. Collectively, resultant data might elucidate the pharmacological mechanism by which the adverse effect of the serotonin syndrome might be precipitated in human mephedrone users, thereby informing the direction of any potential harm management arising from the combination of caffeine with mephedrone. Given the financial constraints of the project, it was therefore determined that examination would be conducted on this receptor exclusively.

The selected mephedrone dose (10.0 mg kg^{-1}) has been shown to elicit sub-maximal changes in locomotor activity (Green *et al.*, 2014) and measurable increases in striatal

5-HT and dopamine efflux (Golembiowska *et al.*, 2016), whilst appearing to have translational relevance to recreational doses used by humans (Shortall *et al.*, 2013a; Shortall *et al.*, 2013b; Green *et al.*, 2014). The caffeine dose was selected as it has been shown to enhance the mephedrone-induced locomotor response, and convert mephedrone-induced hypothermia to hyperthermia (Shortall *et al.*, 2016a) (also Chapter 2, Section 2.4.1.2). Stereotyped behaviour was a key focus for this study and was not examined in prior work by Shortall *et al*., and nor was it quantified by anything other than simple rating scales in Chapter 2. Therefore, to be certain that lateral head weaving could actually be observed following single as opposed to binge-style administration of caffeine and mephedrone at the selected doses a pilot study was performed. These doses were validated in a pilot study where locomotor activity and the frequency of stereotyped behaviours (lateral head weaving and reciprocal forepaw treading) were elevated relative to saline-treated controls (**Figure 3.1**). The selected dose of WAY-100,635 (0.5 mg kg^{-1}) was predicated on a number of observations. Firstly, systemic pre-administration (–30 min, i.p.) at this dose has been shown by this group to attenuate mephedrone-induced hypothermia whilst briefly attenuating concomitant locomotor hyperactivity (Shortall *et al.*, 2016b). Similarly, administered before (–10 min, i.p.) the 5-HT_{1A} agonist 8-OH-DPAT, this dose curtailed drug-induced hypothermia and locomotor hyperactivity, as well as both flat body posture and reciprocal forepaw treading (Kawano *et al.*, 2015). Secondly, at this dose, WAY-100,635 has been shown to counteract the neurochemical effects of 8-OH-DPAT. Specifically, co-administration (s.c.) reversed the effect of 8-OH-DPAT on L-DOPA-induced dyskinesia and striatal glutamate efflux (Dupre *et al.*, 2011), whilst pre-administration (–30 min, s.c.) has elsewhere demonstrably lessened 8-OH-DPAT-induced increases in acetylcholine concentration in the medial prefrontal cortex (Ichikawa *et al.*, 2002). Although WAY-100,635 has been shown to prevent 8-OH-DPAT-induced stereotyped behaviours at lower doses (Forster *et al.*, 1995; Bardin *et al.*, 2001), the current dose was selected as it has been shown by this group to be effective when administered to Lister-hooded rats, and to maintain consistency and allow comparison with previous findings of this group.

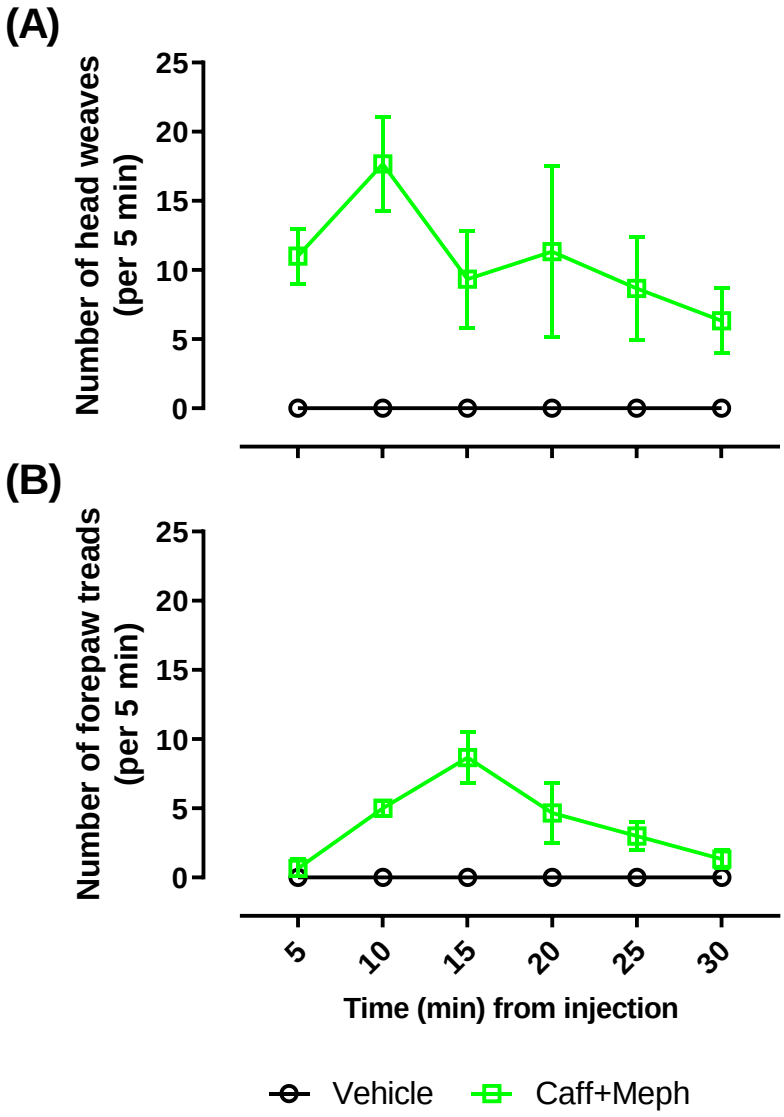

Figure 3.1 Pilot study data validating doses of caffeine (Caff) and mephedrone (Meph) to be used in the present study. The combination of caffeine and mephedrone (10.0 mg kg^{-1} each, i.p.) caused observable lateral head weaves and reciprocal forepaw treads (n = 3 per treatment group), and therefore validated these doses for use in the present experiment. Data are presented as mean ± SEM.

Hypotheses:

We combined these behavioural and physiological assessments with *in vivo* microdialysis to test our hypotheses that:

- The combination of mephedrone plus caffeine would elicit hyperthermia, plus greater hyperactivity, stereotypy and striatal monoamine efflux than either drug alone;
- Pre-treatment with WAY-100,635 would fail to influence mephedrone plus caffeine-induced changes in monoamine efflux, but would prevent the downstream action of endogenous 5-HT on the 5-HT_{1A} receptor and thereby attenuate the hyperactivity and stereotypy produced by combined treatment.

3.2 Materials & methods

3.2.1 Animals

This study used a total of 48 adult male Lister Hooded rats (Charles River UK; 199 – 260 g at the time of surgery, 233 – 299 g at the time of microdialysis), which were maintained under controlled conditions (21.0 ± 2.0 °C; 55 ± 10% humidity; 12 h light-dark cycle, on at 07:00 h) with unlimited access to food and water. Rats were allowed a minimum of seven days to acclimatise to the facility prior to surgery, during which time they were housed in groups of four in individually ventilated cages (GR1800 Double-Decker; Tecniplast, UK) containing sawdust bedding plus environmental enrichment in the form of a cardboard play tube, wooden chew block and paper nesting material. Following surgery rats were individually housed using the same cage type but with the shelf and cardboard tube removed to avoid any potential damage to the microdialysis guide cannula and/or tether. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and ARRIVE guidelines (Percie du Sert *et al.*, 2019), with approval of the University of Nottingham Animal Welfare and Ethical Review Board.

3.2.2 Drugs

(±)-mephedrone-HCl and caffeine ReagentPlus were obtained from Sigma-Aldrich (Poole, UK). N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)-cyclohexanecarboxamide maleate (WAY-100,635) was purchased from Tocris Bioscience (Bristol, UK). All drugs were dissolved in sterile saline vehicle (0.154 M) and doses are quoted as the salt.

3.2.3 Experimental design and drug administration

Drug administration and data acquisition occurred in the light phase (09:00 – 17:00 h), using doses, group sizes and an experimental design **(Fig. 3.1)** chosen to comply with the three Rs of humane animal testing, and for consistency with previous studies (Shortall *et al.*, 2016a; Shortall *et al.*, 2016b).

In summary, guide cannulae were surgically implanted above the striatum and the same day s.c. temperature-sensing microchips (idENTICHIP, with BioThermo, Animalcare; York, UK) were non-surgically implanted into the nape of the neck to allow subsequent monitoring of drug-induced temperature changes, which represents a methodological refinement (as described in Section 2.3.3.1).

Microdialysis probes were inserted into the striatum six days later and data were acquired the next day. Microdialysis samples were then collected (a described in **Fig. 3.2**). Rats were randomly allocated to these pre-treatment x treatment combinations via pseudorandom drawing of lots in a manner that ensured a balanced mix of combinations across test days. Collection of microdialysates continued until 120 min after the second injection and was accompanied by assessment of body temperature, locomotor activity and lateral head weaving. All data were obtained by trained observers. Blinding was achieved as described in Chapter 2 (Section 2.3.1).

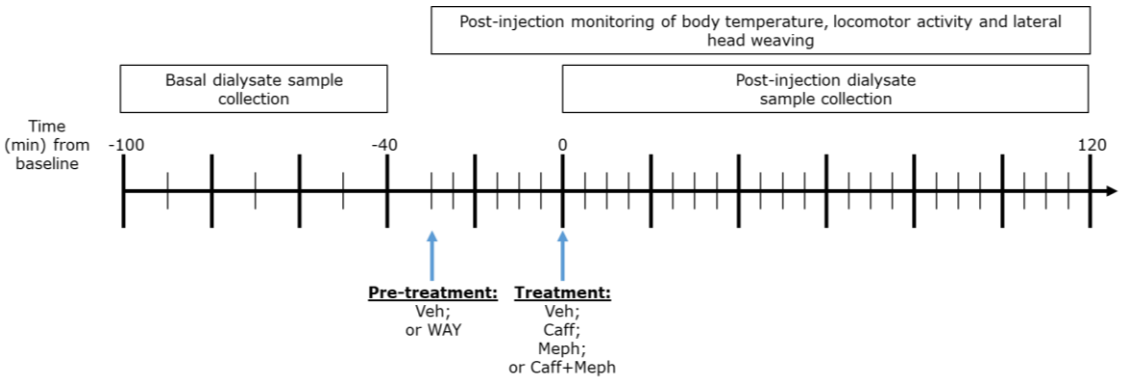

Figure 3.2 Summary of the experimental design. Guide cannulae were stereotaxically implanted above the striatum under isoflurane anaesthesia and microdialysis probes were inserted six days later (without further anaesthesia). The following day singly-housed adult male Lister hooded rats received i.p. pre-treatment with vehicle (saline, 1.0 mL kg^{-1}) or WAY-100,635 (0.5 mg kg^{-1}; WAY) at –30 min, followed by i.p. treatment with vehicle (saline, 1.0 mL kg^{-1}), caffeine (10.0 mg kg^{-1}; Caff), mephedrone (10.0 mg kg^{-1}; Meph), or caffeine plus mephedrone (10.0 mg kg^{-1} each, combined in the same injection) at 0 min (n = 8 per pre-treatment x treatment combination). Striatal microdialysates were collected (at 20 min intervals) from 60 min pre-injection until 120 min after the second injection (indicated by thick black vertical lines). Body temperature (via digital scanning of subcutaneously implanted microchips), locomotor activity (via Ethovision XT 8.5 software) and lateral head weaving (counted from video) were quantified in five minute time bins over the post-injection period.

3.2.4 Surgery and microdialysis

Rats were anaesthetised with isoflurane in O_2 and N_2O and pre-treated with metacam (0.2 mg kg^{-1} s.c.; Boehringer Ingelheim, Germany) to provide post-surgical analgesia. The operative site was shaved and disinfected with chlorhexidine (NHS Supply Chain ROARP/Supply Chain Coordination Ltd; Cotes Industrial Estate, Alfreton) then rats were placed in a stereotaxic frame that had blunt ear bars, with EMLA local anaesthetic cream (AstraZeneca; Cambridge, UK) at the tips, and the incisor bar set 3.3 mm below the interaural line. A heat pad was used to maintain body temperature and a topical

lubricant (Lacrilube; Allergan, UK) to prevent drying of the eyes by surgical lamps. A CMA 12 polyurethane guide cannula (CMA Microdialysis AB, Sweden) **(Fig. 3.4A)** was aseptically implanted just above the striatum using coordinates (AP +1.8, ML ±3.0, DV -3.0 from Bregma) selected from Paxinos and Watson (1983) based on successful measurement of mephedrone-evoked increases in striatal 5-HT and dopamine efflux (Golembiowska *et al.*, 2016) **(Fig. 3.3)**. The guide cannula and a tether (rat collars; Bioanalytical Systems Incorporated; West Lafayette, Indiana, USA)) were secured using stainless steel screws (skull screws, Bossard screws M 1.2 x 3 mm; MDK Fastners, Windsor, Berkshire) and dental cement (Associated Dental Products Ltd, Swindon, UK). Lignocaine local anaesthetic (Castle Veterinary Centre; Nottingham) was applied topically around the sutures (Mersilk, W502; Ethicon, USA) and the area was coated with a plastic dressing (Opsite; Smith and Nephew, UK). Saline (1.0 mL s.c.) was administered to avoid post-operative dehydration. Temperature-sensing microchips were implanted at this point. Post-surgery welfare assessments were completed daily and rats received both analgesia (0.2 mg kg^{-1}, s.c. metacam) and wet mash for the first four days.

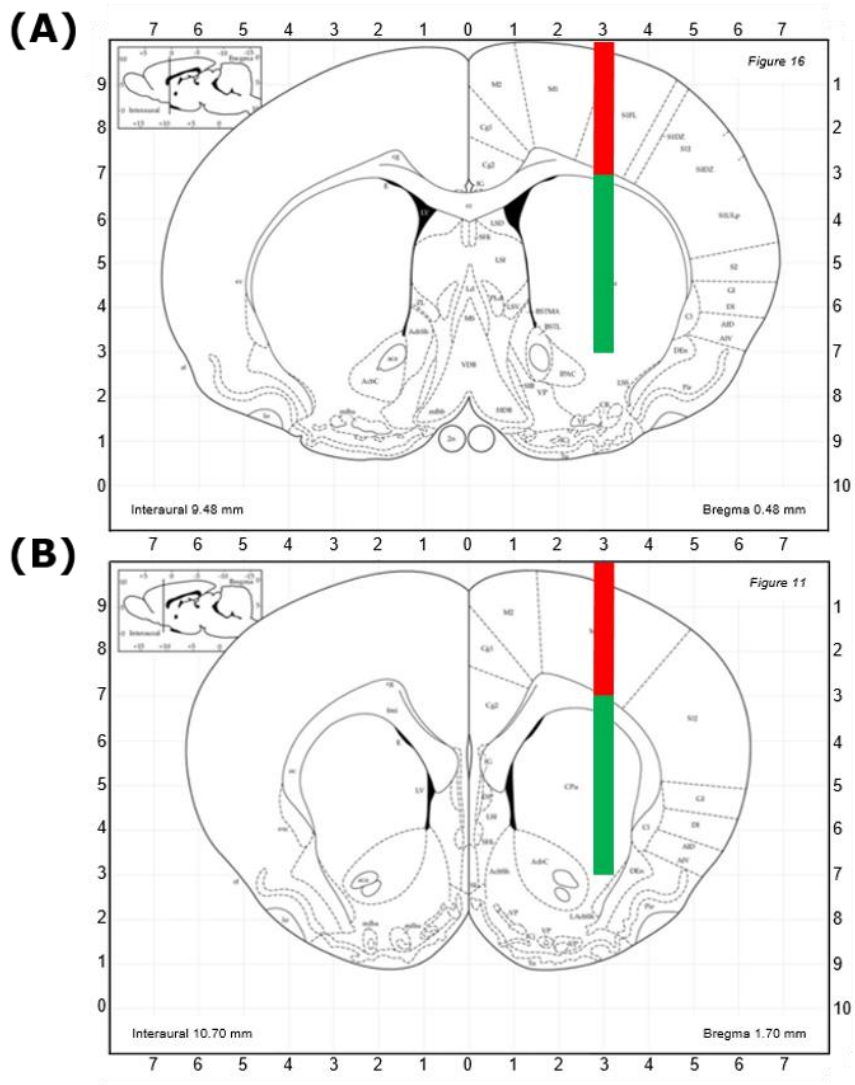

Figure 3.3 Stereotaxic coordinates used for the implantation of microdialysis probe into striatum for *in vivo* microdialysis. Coordinates refer to those used in a previous study of this group (Shortall *et al.*, 2016b) **(A)**; and those used in the present study, based on the findings of others (Kaminska *et al.*, 2018; Noworyta-Sokołowska *et al.*, 2019) **(B)**. Guide cannula and microdialysis probe locations are denoted with red and green bars, respectively. Coordinates for the current study were chosen due to the improved basal levels of monoamines detected by Kaminska *et al.* and Noworyta-Sokołowska *et al*., relative to those previously used by our group (Shortall *et al*.).

Six days post-surgery, a microdialysis probe (CMA 12, 4 mm polyerylethersulpone membrane, 500 µm outer diameter, 3.0 µl internal volume with a 20 kDa molecular cut-off; CMA Microdialysis AB) **(Fig. 3.4B)** was inserted through the guide cannula under brief manual restraint but no further anaesthesia. Probe selection was based on previous studies of dopamine and 5-HT efflux (Shortall *et al.*, 2016b; Kohli *et al.*, 2019). The probe was connected to a microinfusion pump (Harvard Scientific, USA) using FEP microdialysis tubing (Linton Instrumentation; Norfolk) and a liquid swivel (CMA Microdialysis AB) to allow unrestricted movement. Rats were individually placed in transparent polycarbonate round-bottomed bowls (36 cm diameter with bedding, 36 cm height with a side access panel to facilitate temperature scanning; BASi; IN, USA) **(Fig. 3.4C)** containing sawdust bedding, food and water. Artificial cerebrospinal fluid (125.0 mM NaCl, 13.5 mM $NaHCO_3$, 1.25 mM KCl, 0.2 mM NaH_2PO_4, 0.9 mM Na_2HPO_4, 0.3 mM Na_2SO_4, 0.5 mM $MgCl_2$, 1.2 mM $CaCl_2H_2O$ adjusted to pH 7.4 with phosphoric acid; Kohli *et al.*, 2019) was continuously perfused at 1.0 µl min^{-1}. The following day the flow rate was increased to 1.2 µl min^{-1} and under these conditions the dead space from the probe to the collection tube resulted in a delay of 3 min 31 seconds which was corrected for before sample collection. Dialysate samples were collected at 20 min intervals from 60 min pre-injection until 120 min after the second injection, into 5.0 µL of 0.1 M perchloric acid (PCA) containing 0.03% sodium metabisulfite, and stored on dry ice and then at –80°C until analysis by HPLC-ED.

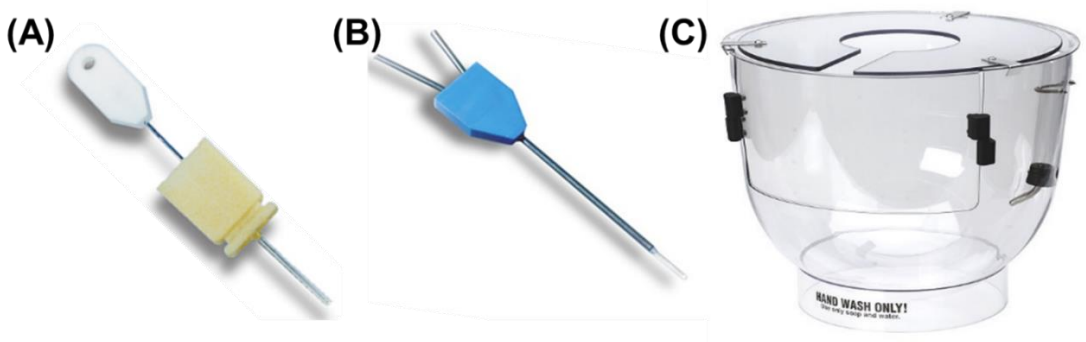

Figure 3.4 Guide cannula (A), microdialysis probe (B) and bowl used for *in vivo* microdialysis (C). (Note: not to scale).

3.2.5 Temperature recording

Body temperature was recorded with minimal disturbance to the animal by scanning the s.c. microchip with a hand-held digital chip reader at 5 min intervals from –30 to 120 min.

3.2.6 Locomotor activity, observations of behaviour, and counts of lateral head weaving

Locomotor activity was measured across the duration of the microdialysis procedure and was recorded by Ethovision XT 8.5 software, which computed distance moved (m). Observations of *in situ* behaviour were conducted every 5 min, and scored on a scale of 0 to 15 (as described in Section 2.3.3.2). The number of head weaves (one count equalling a motion toward both left and right and a resetting in the middle) in 5 min time bins was counted by five final year undergraduate project students and the author from video files. Each project student observed a total of 8 videos (comprising at least one, but no more than two, animals of each treatment group), and allocated spot checks of 5 min per video were conducted by the project supervisor, who was also unaware of treatment.

3.2.7 Histological verification of probe position

Rats were killed by i.p. injection of euthatal (1.0 mL) before cervical dislocation. Brains were rapidly removed and hemisected on a refrigerated dissection table (BC72: Osborne refrigeration, UK, 4.0 °C), immerse fixed in 4% paraformaldehyde (4.0 °C overnight), cryopreserved in 0.1 M phosphate buffered saline containing 30 % (w/v) sucrose (4.0 °C overnight) then snap-frozen in isopentane on dry ice. Probe location was verified by slicing of the left hemisphere into 100 µm thick sections, and determined with reference to Paxinos & Watson's stereotaxic atlas (Paxinos &

Watson, 1983), on a freezing microtome (Anglia Scientific; Cambridge, UK). Images of slices were captured by a mobile phone camera (Huawei P Smart 2020; 415 pixels per inch) **(Fig. 3.5)**.

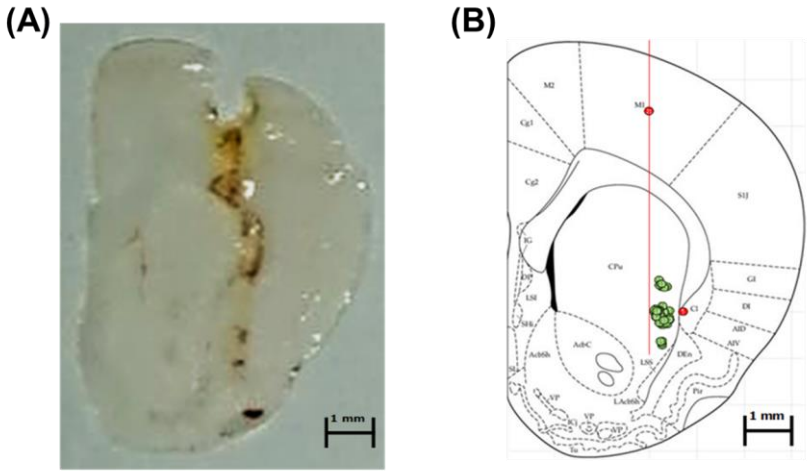

Figure 3.5 Histological validation of probe position. Representative brain section showing an example of the probe tract **(A)** and atlas image **(B)** with symbols marking the approximate position of the probe tip in each of the 48 rat brains, with appropriate scale bars. Desired probe position indicated by red line. Successful location of probe in the striatum is indicated by green data points (n = 46). Unsuccessful location of probe outside the striatum is indicated by red data points (n = 2).

3.2.8 Neurochemical detection by HPLC-ED

Each sample was kept on dry ice and then stored at –80 ^{o}C until planned analysis of dopamine, 5-HT and their major metabolites by high performance liquid chromatography-electrochemical detection, as described in previous publications from this group (King *et al.*, 2009; Shortall *et al.*, 2013b; Shortall *et al.*, 2016b; Kohli *et al.*, 2019).

Despite spending a period of eight months (interrupted by five months of working restrictions due to COVID-19 pandemic) attempting to optimise the HPLC-ED apparatus to analyse these samples, it proved not possible within the financial and time constraints of the present thesis. **Appendix I** summarises the key changes made in the interests of this optimisation and illustrates that ongoing issues with the signal-noise ratio prevented analysis of dialysates.

It was intended that HPLC-ED would be performed according to previous methods (Rodsiri *et al.*, 2011; Shortall *et al.*, 2016b; Kohli *et al.*, 2019). Briefly, dialysate samples were to be injected at a volume of 15.0 µL onto a Luna® C18 3.0 µm 100 Å, LC Column (Phenomenex; Cheshire, UK) using a Perkin Elmer AS200 autosampler (Antec Leyden; The Netherlands). Initial mobile phase composition was 20.0 mM potassium dihydrogen phosphate (KH_2PO_4), 20.0 mM sodium acetate (NaAc), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.15 mM octanesulfonic acid (OSA) and 4.0% methanol (pH 3.89) would be circulated throughout the system at a flow rate of 0.05 ml min^{-1} via and Ultimate 3000 pump (Dionex) at a constant pressure of *circa* 1000 psi. This composition was based on a previous study of this group which successfully measured dopamine, 5-HT, and the metabolites DOPAC, HVA and 5-HIAA (Kohli *et al.*, 2019). Analytes were to be detected with a DECADE II SDC Detector I (Antec; Leyden; The Netherlands) using an Antec VT-03 cell with a glassy carbon 2.0 mm working electrode at +0.59 V with an *in situ* Ag/AgCl ISAAC reference electrode.

Pooled standards were to be prepared daily from fresh stock solutions (1×10^{-4} M) and diluted with 0.05 M PCA to concentrations of 1×10^{-8}, 1×10^{-7} and 2×10^{-7} M. These pooled standards were to be injected at the start of each day and following every 11 samples. Quantification was to be performed against standards using Clarity software (Data Apex).

3.2.9 Statistical analyses

All statistical analyses were conducted using GraphPad Prism (v 8.4.2) or SPSS (v 26) software. Data were checked for normality (with Kolgomorov-Smirnov or Shapiro-Wilk's tests) and homogeneity of variance (with Mauchly's test of sphericity) before use of parametric tests. Locomotor activity and changes in body temperature were analysed by three-way repeated measures ANOVA followed by Tukey's multiple comparisons post-hoc test. Because this study employed an unbalanced design (lacking the combination of either WAY-100,635 with either caffeine or mephedrone alone, to avoid the unnecessary use of an additional 16 rats) these analyses were applied separately to two different subgroups. The first (to assess interactions between caffeine and mephedrone) included only vehicle pre-treated rats, with caffeine and mephedrone as separate between-subjects factors and time as a within-subjects repeated measure. The second (to assess whether the combined effect of caffeine plus mephedrone was sensitive to 5-HT_{1A} receptor antagonism) included only vehicle or caffeine plus mephedrone-treated rats, with WAY-100,635 pre-treatment and combined caffeine plus mephedrone treatment as separate between-subjects factors and time as a within-subjects repeated measure. Importantly, body temperature change at the 0 min baseline time point (which had a mean and variance of zero) were not included in any statistical analyses. Because variance at the remaining time points differed between treatment combinations, a Greenhouse-Geisser correction was applied to the ANOVAs. Behavioural observation scores were categorical, not linear, so were analysed using non-parametric Mann-Whitney U tests. Stereotyped head weaving counts were not normally distributed so were analysed using non-parametric Kruskall Wallis tests with Dunn's multiple comparisons post-hoc (due to the absence of any non-parametric equivalent to a two-way ANOVA), again applied separately to two different subgroups. In each case $P < 0.05$ was considered statistically significant. Behavioural observation scores and head weaving data are presented as median ± interquartile range and all other data are presented as mean ± SEM.

3.3 Results

3.3.1 Changes in body temperature

The mean basal subcutaneous temperature immediately prior to the first injection was 37.28 ± 0.05°C, which is consistent with other basal values using the same technique in our laboratory (e.g. 37.22 ± 0.10°C; Goh et al. unpublished observations). There were no between-group differences in baseline temperatures at 0 min, immediately prior to the second injection (vehicle 37.31 ± 0.13 °C, WAY-100,635 alone 37.68 ± 0.14 °C, caffeine alone 37.45 ± 0.17 °C, mephedrone alone 37.55 ± 0.16 °C, caffeine plus mephedrone 37.55 ± 0.17 °C, WAY-100,635 with caffeine plus mephedrone 37.70 ± 0.09 °C; $F_{(5, 42)} = 0.964$, $P = 0.451$).

There were main effects of time ($F_{(4.58, 128.17)} = 3.356$, $P = 0.009$) and caffeine ($F_{(1,28)} = 10.857$, $P = 0.003$), together with time x caffeine ($F_{(4.58, 128.17)} = 5.320$, $P < 0.001$) and time x mephedrone ($F_{(4.58, 128.17)} = 3.013$, $P = 0.016$) interactions. Caffeine or mephedrone alone each induced short-term hyperthermia, which reached significance for 30 min out of the 120 min monitoring period in the case of caffeine (intermittently between 50 and 110 min post-injection), and 15 min out of the same monitoring period for mephedrone (intermittently from 50 to 75 min post-injection). Although there were no caffeine x mephedrone ($F_{(1,28)} = 0.499$, $P = 0.486$) or time x caffeine x mephedrone ($F_{(4.58, 128.17)} = 1.530$, $P = 0.190$) interactions the co-administration of caffeine with mephedrone produced a more prolonged hyperthermia than either drug alone, which was evident for 70 of the 120 min and of note greater than that induced by mephedrone alone from 105 to 120 min post-injection **(Fig. 3.6A)**.

There was no main effect of WAY-100,635 ($F_{(1,28)} = 0.032$, $P = 0.859$) nor any time x WAY-100,635 interaction ($F_{(3.89,109.04)} = 0.898$, $P = 0.466$). The main effect of caffeine plus mephedrone ($F_{(1,28)} = 42.128$, $P < 0.001$) did not interact with WAY-100,635 ($F_{(1,28)}$

= 3.022, P = 0.093) and the time x caffeine plus mephedrone interaction ($F_{(3.89,109.04)}$ = 17.829, P < 0.001) also failed to interact with WAY-100,635 ($F_{(3.89,109.04)}$ = 0.454, P = 0.764). Thus, significant hyperthermia occurred in caffeine plus mephedrone-treated rats in both the presence and absence of WAY-100,635, although the onset of this hyperthermia was observed 40 minutes earlier in rats pre-treated with the antagonist **(Fig 3.6B)**.

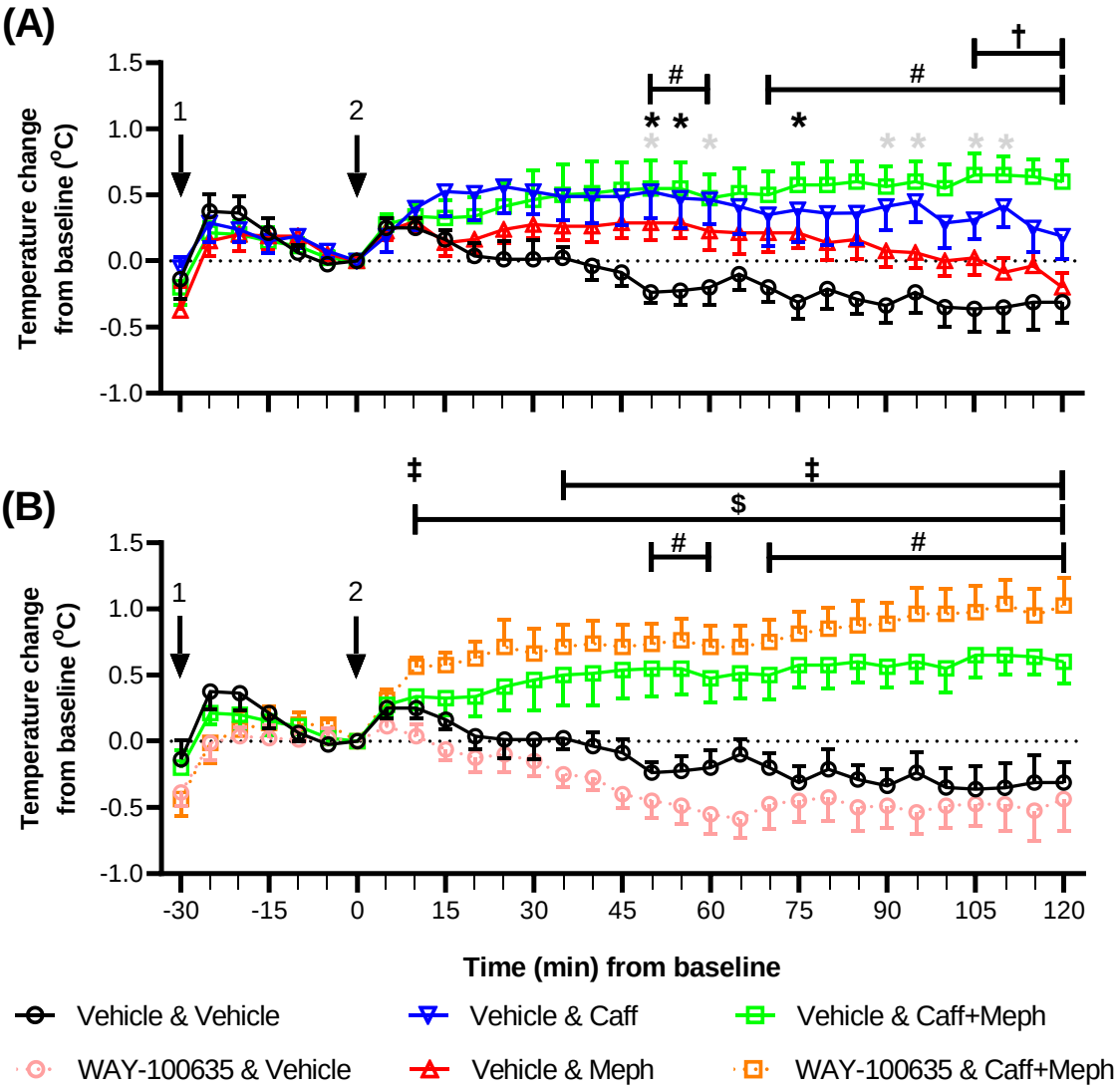

Figure 3.6 Impact of (A) caffeine co-administration on mephedrone-induced hyperthermia and (B) the 5-HT$_{1A}$ receptor antagonist WAY-100,635 on the combined hyperthermic response to caffeine plus mephedrone. Locomotor activity was measured across the duration of the microdialysis procedure and was recorded by

Ethovision XT 8.5 software, which computed distance moved (m). Mean ± SEM change in subcutaneous temperature (from the baseline value at 0 min) during simultaneous striatal microdialysis in singly-housed adult male Lister hooded rats that received i.p. pre-treatment with vehicle (saline, 1.0 mL kg^{-1}) or WAY-100,635 (0.5 mg kg^{-1}; WAY) at –30 min (denoted as "**1**" on graph), followed by i.p. treatment with vehicle (saline, 1.0 mL kg^{-1}), caffeine (10.0 mg kg^{-1}; Caff), mephedrone (10.0 mg kg^{-1}; Meph), or caffeine plus mephedrone (10.0 mg kg^{-1} each combined in the same injection; Caff + Meph) at 0 min (denoted as "**2**" on graph) (n = 8 per pre-treatment x treatment combination). *P < 0.05 Vehicle & Caff, *****P < 0.05 Vehicle & Meph, and #P < 0.05 Vehicle & Caff + Meph versus Vehicle & Vehicle; **†**P < 0.05 Vehicle & Caff + Meph versus Vehicle & Meph; **‡**P < 0.05 WAY-100,635 & Caff + Meph versus Vehicle & Vehicle; $$P$ < 0.05 WAY-100,635 & Caff + Meph versus WAY-100,635 & Vehicle (Tukey's multiple comparison post-hoc following three-way repeated-measures ANOVA, applied separately to data in panels A and B).

3.3.2 Locomotor activity

There were main effects of time ($F_{(30, 840)}$ = 12.957, P < 0.001), caffeine ($F_{(1, 28)}$ = 11.028, P < 0.01) and mephedrone ($F_{(1, 28)}$ = 6.786, P < 0.05), together with time x caffeine ($F_{(30, 840)}$ = 2.778, P < 0.001) and time x mephedrone ($F_{(30, 840)}$ = 2.437, P < 0.001) interactions. Caffeine or mephedrone alone each caused an increase in locomotor activity, which reached significance at 60 min in the case of caffeine, and between 20 and 25, as well as 75 min in the case of mephedrone. Although there were no caffeine x mephedrone ($F_{(1, 28)}$ = 0.846, P = 0.366) or time x caffeine x mephedrone ($F_{(30, 840)}$ = 0.588, P = 0.962) interactions, the co-administration of caffeine with mephedrone produced a more prolonged increase in locomotor activity than either drug alone, which was evident for 55 of the 120 min and of note greater than that induced by mephedrone alone at 55 and 120 min post-injection **(Fig. 3.7A)**.

There was no main effect of WAY-100,635 ($F_{(1, 28)}$ = 0.133, P = 0.718) nor any time x WAY-100,635 interaction ($F_{(30, 840)}$ = 0.555, P = 0.975). The main effect of caffeine plus mephedrone ($F_{(1, 28)}$ = 21.023, P < 0.001) did not interact with WAY-100,635 ($F_{(1, 28)}$ = 0.502, P = 0.484) and the time x caffeine plus mephedrone interaction ($F_{(30, 840)}$ = 7.539, P < 0.001) also failed to interact with WAY-100,635 ($F_{(30, 840)}$ = 1.345, P = 0.103). Thus, significant hyperlocomotion occurred in caffeine plus mephedrone-treated rats in both the presence and absence of WAY-100,635, although the onset of increased locomotion appeared to be delayed (by 5 min) in the presence of the 5-HT_{1A} antagonist **(Fig. 3.7B)**.

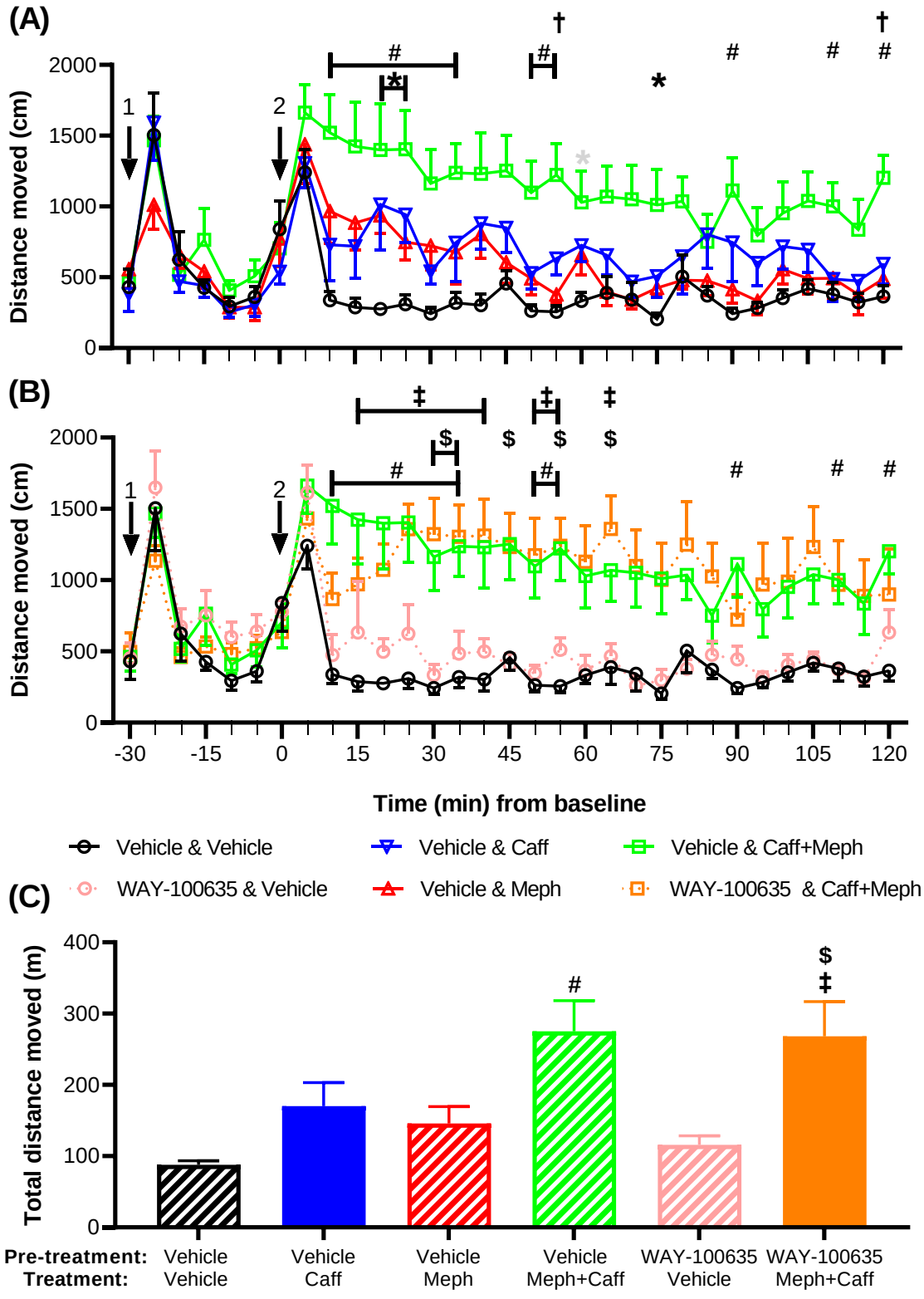

Figure 3.7 Effect of (A) caffeine co-administration on mephedrone-induced hyperactivity, (B) the 5-HT$_{1A}$ receptor antagonist WAY-100,635 on the combined locomotor response to caffeine plus mephedrone, and (C) total distance moved by all groups. Mean ± SEM distance moved during simultaneous striatal microdialysis in

singly-housed adult male Lister hooded rats that received i.p. pre-treatment with vehicle (saline, 1.0 mL kg^{-1}) or WAY-100,635 (0.5 mg kg^{-1}; WAY) at –30 min (denoted as "**1**" on graph), followed by i.p. treatment with vehicle (saline, 1.0 mL kg^{-1}), caffeine (10.0 mg kg^{-1}; Caff), mephedrone (10.0 mg kg^{-1}; Meph), or caffeine plus mephedrone (10.0 mg kg^{-1} each combined in the same injection; Caff + Meph) at 0 min (denoted as "**2**" on graph) (n = 8 per pre-treatment x treatment combination). $^{*}P < 0.05$ Vehicle & Caff, $^{**}P < 0.05$ Vehicle & Meph, and $^{\#}P < 0.05$ Vehicle & Caff + Meph versus Vehicle & Vehicle; $^{\dagger}P < 0.05$ Vehicle & Caff + Meph versus Vehicle & Meph; $^{\ddagger}P < 0.05$ WAY-100,635 & Caff + Meph versus Vehicle & Vehicle; $^{\$}P < 0.05$ WAY-100,635 & Caff + Meph versus WAY-100,635 & Vehicle (Tukey's multiple comparison post-hoc following three-way repeated-measures ANOVA, applied separately to data in panels A and B).

3.3.3 Stereotyped behaviour

3.3.3.1 Behavioural observations

Mephedrone or caffeine alone elicited an increase in behavioural observation scores 5 and 25 minutes post-injection, respectively, lasting for 25 and 40 of the 120 minute post-injection period, respectively **(Fig. 3.8A)**. The combination of mephedrone and caffeine caused a further increase in these scores, relative to vehicle-treated rats, for 115 of the 120 minute post-injection period, eliciting an increase in behavioural activity 20 minutes earlier than caffeine alone, and lasting for 90 minutes longer than mephedrone alone.

Pre-treatment with WAY-100,635 failed to prevent the increase in behavioural observation scores from 5 minutes post-treatment, though WAY-100,635 did seem to curtail behavioural activity for the final 10 minutes of the 120 post-treatment period **(Fig. 3.8B)**.

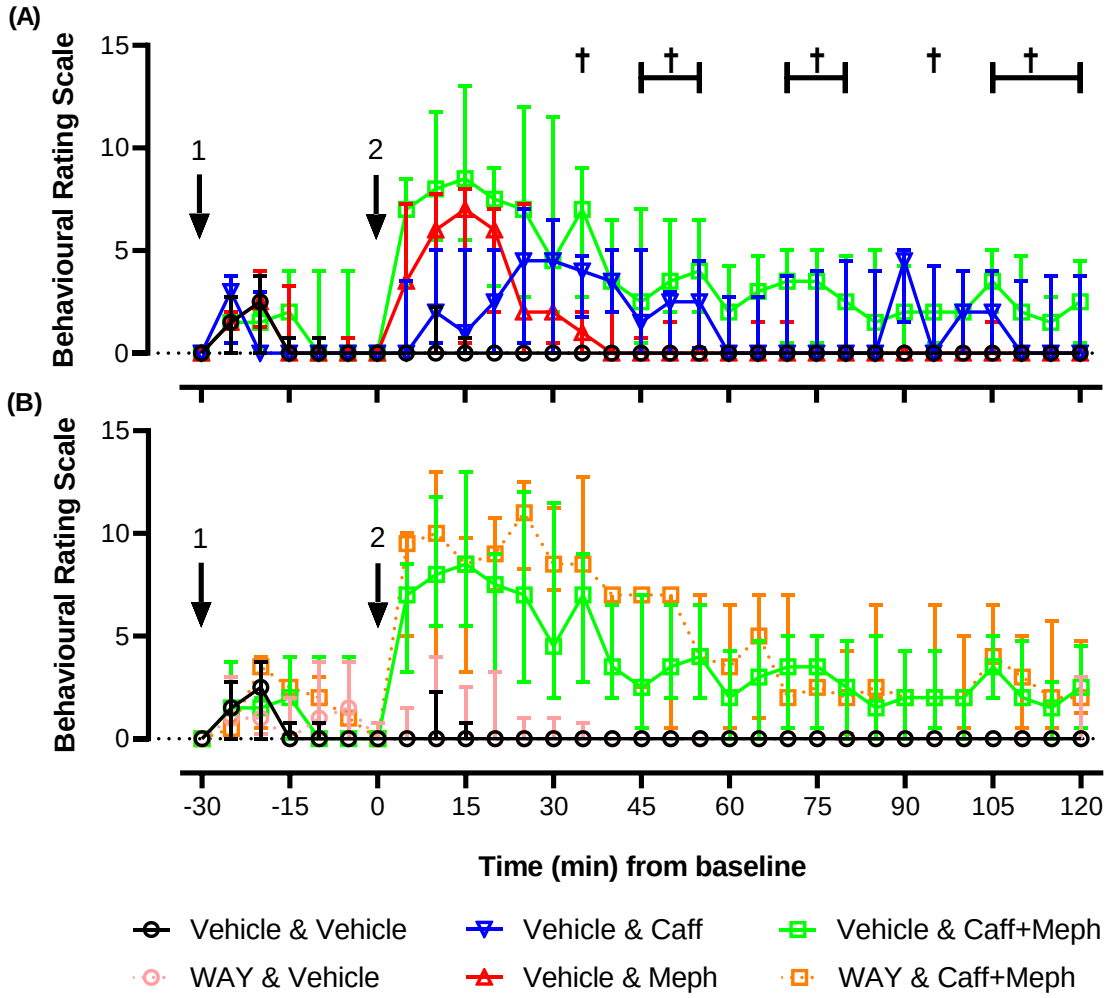

Figure 3.8 Effect of (A) caffeine, mephedrone and their combination on the behavioural rating determined by a treatment-blind observer, and of (B) the 5-HT$_{1A}$ receptor antagonist WAY-100,635 on the combined response to caffeine plus mephedrone. Median ± interquartile range, for behavioural observations made over a 120 min period during simultaneous striatal microdialysis. Singly-housed adult male Lister hooded rats received i.p. pre-treatment (–30 min) with vehicle (saline, 1.0 mL kg^{-1}) or WAY-100,635 (0.5 mg kg^{-1}) (denoted as "**1**" on graph) then i.p. treatment with vehicle (saline, 1.0 mL kg^{-1}), caffeine (10.0 mg kg^{-1}; Caff), mephedrone (10.0 mg kg^{-1}; Meph), or caffeine plus mephedrone (10.0 mg kg^{-1} each combined in the same injection; Caff + Meph; n = 8 per pre-treatment x treatment combination) (denoted as "**2**" on graph). Scores are for the 120 min period following this second injection. Scores were compared using planned pairwise Mann-Whitney U tests, analysing differences between groups at each time point. $^{*}P < 0.05$ Vehicle & Caff, $^{*}P < 0.05$ Vehicle & Meph,

and #*P* < 0.05 Vehicle & Caff + Meph versus Vehicle & Vehicle; †*P* < 0.05 Vehicle & Caff and **†***P* < 0.05 Vehicle & Meph versus Vehicle & Caff + Meph; $*P* < 0.05 WAY-100,635 & Caff + Meph versus WAY-100,635 & Vehicle.

3.3.3.2 Lateral head weaving

Lateral head weaving was absent in rats that received only vehicle, WAY-100,635 or caffeine, but exhibited by a proportion of rats in each of the three mephedrone-treated groups **(Fig. 3.9)**, particularly in the first 30 min post-injection (data not shown). There appear to be responders and non-responders in each of these groups but irrespective of this mephedrone-induced increases in total head weaving only reached significance after combination with caffeine, and the combined effect was not further modified by pre-treatment with WAY-100,635 **(Fig. 3.9)**.

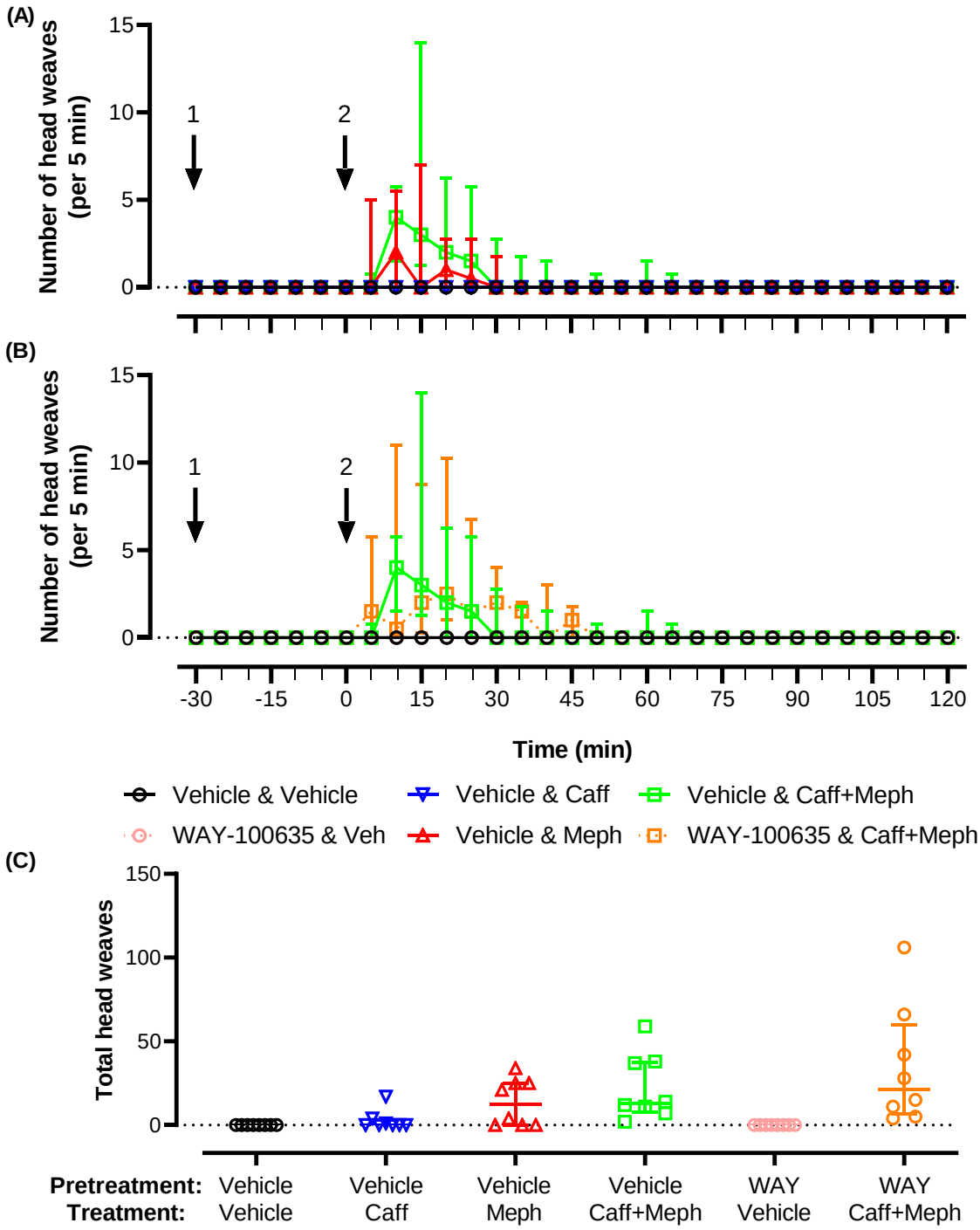

Figure 3.9 Impact of caffeine co-administration on mephedrone-induced lateral head weaving, and of the 5-HT$_{1A}$ receptor antagonist WAY-100,635 on the combined response to caffeine plus mephedrone. Median ± interquartile range, for the time course **(A, B)** and total number of lateral head weaves **(C)** over a 120 min period during simultaneous striatal microdialysis. Singly-housed adult male Lister hooded rats received i.p. pre-treatment (–30 min) with vehicle (saline, 1.0 mL kg^{-1}) or WAY-100,635 (0.5 mg kg^{-1}; WAY) then i.p. treatment with vehicle (saline, 1.0 mL kg^{-1}), caffeine (10.0

mg kg^{-1}; Caff), mephedrone (10.0 mg kg^{-1}; Meph), or caffeine plus mephedrone (10.0 mg kg^{-1} each combined in the same injection; Caff + Meph; n = 8 per pre-treatment x treatment combination). Counts are for the 120 min period following this second injection. Data from groups that did not receive mephedrone had a median and variance of zero and were therefore not included in statistical analysis. The remaining three groups were compared using planned pairwise Mann-Whitney U tests (Vehicle & Meph versus Vehicle & Caff + Meph (U = 21.0, P = 0.279); Vehicle & Caff + Meph versus WAY-100,635 & Caff + Meph (U = 26.5, P = 0.574)).

3.4 Discussion

It was hypothesised that, as in Chapter 2, the co-administration of mephedrone and caffeine would elicit hyperthermia, locomotor hyperactivity, and stereotyped behaviours, and indeed each of these predicted effects were observed. It was also hypothesised that pre-treatment with the 5-HT_{1A} antagonist WAY-100,635, without altering hyperthermia or 5-HT efflux in the striatum, would prevent stereotyped behaviours and attenuate locomotor hyperactivity following this combination. However, WAY-100,635 failed to prevent these stereotyped behaviours. Meanwhile, WAY-100,635 had no effect on the extent of hyperthermia (when the WAY-100,635 and combination was compared to the vehicle and combination treatment), but the onset of significant differences versus the vehicle and vehicle control group occurred sooner following WAY-100,635 and combination than vehicle and combination. The absence of dialysate data makes it not possible at this juncture to ascertain the neurochemical effects of combined versus separate drug treatment.

Individually, both mephedrone and caffeine produced a transient hyperthermia following administration, with body temperature returning to baseline within 75 and 110 minutes, respectively. The combination of the two drugs elicited a more pronounced hyperthermia relative to either drug alone, which remained elevated for the duration of testing. In rats, mephedrone-induced hyperthermia is typically

observed following administration at high doses (den Hollander *et al.*, 2014a; Grecco & Sprague, 2016; Zona *et al.*, 2016), and at normal (Baumann *et al.*, 2012) to high ambient room temperature (Hadlock *et al.*, 2011). Despite subtle methodological differences, these findings were consistent with those of Chapter 2, as well as some (but not all) previous findings of our group (Shortall *et al.*, 2016a). These methodological differences included the increased dose of mephedrone used (10.0 mg kg^{-1} versus 7.5 mg kg^{-1} in Chapter 2) and the different environmental conditions in which the experiment was conducted: measurements of body temperature were taken every five minutes, versus every 15; during testing, rats were individually-housed, versus group-housed; rats received the drug 30 minutes after a previous injection; and measurements were taken in the relatively novel arenas to which rats had acclimatised overnight, versus the home cage in which rats spent several weeks prior to testing. Further, the day before measurements of body temperature, a microdialysis probe was implanted into the guide cannula and the rat connected to a tether and aCSF perfusion tubes. Whilst this procedure is ethically permissible, stress levels may conceivably have been different to those experienced in the home cage during the previous experiment. However, the current observation of mephedrone-induced hyperthermia is at odds with some other findings of the group. Shortall *et al.* (2013) found decreases in both rectal (core) and tail (skin) temperature following acute administration of the same dose used here to rats placed in individual Perspex arenas for the 160 min duration of the study. Likewise, although our group previously showed that mephedrone effected a hypothermic response when administered at the same dose as used presently, it is important to note that body temperature in this earlier study was measured via different means. The hypothermic response to mephedrone, persisting for two hours post-administration (Shortall *et al.*, 2013a), was assessed via tail temperature, and is therefore representative of changes in skin temperature that are consistent with anecdotal evidence of unpleasant peripheral changes in human users (Ahmed *et al.*, 2010; Dick & Torrance, 2010; Dargan *et al.*, 2011; Schifano *et al.*, 2011; Vardakou *et al.*, 2011). Similarly, the short-lasting hypothermic effect on rectal temperature, observed by Shortall *et al.* shortly after administration, may differ from the hyperthermic effect on subcutaneous body temperature observed here, as a consequence of differences in data acquisition, as

well as other methodological differences. The consistency of this hyperthermic response in spite of methodological differences indicates the robustness of the effects of this drug combination in rats, suggesting enhanced translational relevance of these particular effects to those observed in human users.

Peripheral thermogenesis, such as of skeletal muscle and white fat, is in large part mediated via noradrenaline/norepinephrine (Bianco *et al.*, 1988; Rubio *et al.*, 1995a; Rubio *et al.*, 1995b), whilst regulation of peripheral body temperature, including through conservation of heat by vasoconstriction, is mediated via vascular α_1- and β-adrenoceptors. Antagonism of α_1-adrenoceptors with multiple ligands has previously been shown to convert the hyperthermic effect of MDMA in mice to a biphasic response characterised by an initial hypothermia followed by hyperthermia, and suggest particular relevance of the α_{1A} and α_{1D} receptor subtypes in this hyperthermia (Bexis & Docherty, 2008). Similarly in rats, the central and peripheral hyperthermic response to MDMA was attenuated by α_1 and β_3 antagonism, respectively (Sprague *et al.*, 2003). Coupled with the prevention of mephedrone-induced hyperthermia by antagonism α_1- and β-adrenoceptors (Zona *et al.*, 2016), and the observation that mephedrone-induced hypothermia was prolonged by α_1 antagonism (Shortall *et al.*, 2013a), these literature support the contention that the hyperthermic effect of mephedrone observed in this present chapter, (as well as following repeated administration of mephedrone in Chapter 2) may be mediated at least in part by activation of the α_1- and β-adrenoceptors.

As in Chapter 2, mephedrone acutely effected a rapid onset of locomotor hyperactivity, the magnitude and duration of which was enhanced by caffeine, co-administration of which elicited a more rapid onset which remained transiently elevated for the duration of testing, and was significantly enhanced relative to mephedrone-treated rats around one and two hours post-administration, likely as a downstream effect of adenosine receptor antagonism.

Antagonism of 5-HT_{1A} receptors with WAY-100,635 has previously been shown to prevent mephedrone- (Shortall *et al.*, 2016b) and MDMA-induced hypothermia (Rusyniak *et al.*, 2007). Likewise, increased 5-HT_{1A} receptor density of the frontal cortex and hypothalamus one week following MDMA administration (30.0 mg kg^{-1}, i.p.) was associated with potentiation of the hypothermic response following administration of 8-OH-DPAT (1.0 mg kg^{-1}, s.c.) (Aguirre *et al.*, 1998). As the combination of mephedrone and caffeine presently elicited hyperthermia, it is perhaps not unexpected that WAY-100,635 failed to curtail this effect. Rather, the hyperthermia observed here as a response to combination treatment is likely mediated by downstream effects, such as on the α_1- and β-adrenoceptors (Shortall *et al.*, 2013a; Zona *et al.*, 2016), as well as following the binding of mephedrone and endogenous 5-HT to 5-HT_{2A} receptors. Nonetheless, it is interesting to note that pre-treatment with the 5-HT_{1A} antagonist served to speed the onset of the hyperthermic response following combination treatment, with elevations noted 40 minutes earlier than in rats pre-treated with vehicle. This might be attributable to binding of WAY-100,635 not only to postsynaptic 5-HT_{1A} receptors, but also to somatodendritic autoreceptors in the median and dorsal raphe nuclei. Activation of these cell body autoreceptors would normally inhibit 5-HT release in forebrain projection areas, therefore 5-HT_{1A} autoreceptor blockade by WAY-100,635 could conceivably disinhibit 5-HT release and actually augment the combined neurochemical, thermoregulatory and behavioural effects of mephedrone and caffeine. The implication of increased availability of synaptic 5-HT in the elicitation of hyperthermia is suggested by the study of SERT-knockout rats, in which WAY-100,635 has previously been shown to dose-dependently elicit hyperthermia (Olivier *et al.*, 2008a). Extracellular 5-HT content is regulated chiefly by SERT (Blakely *et al.*, 1991; Murphy *et al.*, 1998), and deletion of the gene encoding this transporter in rats (Smits *et al.*, 2004) has been shown to precipitate both decreases and increases in tissue levels and extracellular levels of hippocampal 5-HT, respectively (Homberg *et al.*, 2007; Olivier *et al.*, 2008b). As a consequence of SERT's deletion, the increased availability of synaptic 5-HT appears to be a prerequisite for WAY-100,635-induced hyperthermia, supporting the present suggestion that this antagonist contributed to enhancing the hyperthermic effect observed presently. In sum, it would suggest that both mephedrone and endogenous

5-HT (elevated via independent mechanisms by mephedrone and caffeine, as well as by WAY-100,635) might have acted via activation of 5-HT_{2A} receptors to elicit the presently observed hyperthermia. This effect would likely have been enhanced further by activation of α_1- and β-adrenoceptors, the former of which mephedrone also exhibits binding affinity in the millimolar range (Simmler *et al.*, 2013).

Blockade of the 5-HT_{1A} receptor rendered a brief delay in the onset of increased locomotor activity following combination treatment, but otherwise failed to attenuate the effect. A similar effect was noted previously by this group, whereby WAY-100,635 briefly attenuated mephedrone-induced locomotor hyperactivity shortly after mephedrone administration (Shortall *et al.*, 2016b), suggesting at least a minor role for activation of this receptor in the hyperactivity observed in response to both mephedrone and mephedrone's combination with caffeine. Nonetheless, mephedrone's hyperlocomotor effect in rats appears to be mediated in larger part via downstream effects on other receptors, notably 5-HT_{2A} (Lopez-Arnau *et al.*, 2012), 5-HT_{1B} (Shortall *et al.*, 2016b) and D_1 (Lisek *et al.*, 2012; Nguyen *et al.*, 2016), whilst caffeine enhances this effect via adenosine receptor antagonism (Kim & Palmiter, 2003; Chen *et al.*, 2010; Taura *et al.*, 2018) and indirect action at co-localised D_2 receptors of striatopallidal GABA neurons (Svenningsson *et al.*, 1999). Implication of these former receptors is much akin to the pharmacological mechanisms underlying the locomotor stimulating effect of MDMA, which has previously been attenuated by antagonism of the 5-HT_{1B} and 5-HT_{2A} receptors with GR127935 and ketanserin, respectively (McCreary *et al.*, 1999; Fletcher *et al.*, 2002). Regarding the involvement of dopamine receptors, the effect of locomotor hyperactivity following mephedrone has been inhibited by blockade of D_1 receptors with SCH 23390 (Lisek *et al.*, 2012), consistent with prevention of this effect in response to both cocaine (Cabib *et al.*, 1991) and MDMA (Benturquia *et al.*, 2008) following antagonism of this receptor, and consistent also with the established importance of both dopamine and dopamine receptors in mediating locomotor activity (Missale *et al.*, 1998).

Aside from its action at 5-HT_{1A} receptors, WAY-100,635 has also been shown to function as a full agonist at dopamine D_4 receptors, where its' major metabolite, WAY-100,634, also behaves as a near full agonist (EC_{50} = 9.7 ± 2.2, and 0.65 ± 0.2 nM, respectively, in HEK-293 cells stably expressing human $D_{4.4}$ receptors) (Chemel *et al.*, 2006). Although no data were available on the timing of WAY-100,634 metabolism in rats *in vivo*, this metabolite has been detected in human and cynomolgus monkey plasma within, respectively five and thirty minutes of i.v. administration of [*O*-methyl-^{11}C]-WAY-100,635 (Osman *et al.*, 1996). Activation of D_4 receptors, such as with A-412997, has been shown to increase locomotor activity in rats (Browman *et al.*, 2005; Woolley *et al.*, 2008). However, given that our group previously demonstrated that WAY-100,635 did not enhance mephedrone-induced hyperactivity at the doses used presently (Shortall *et al.*, 2016b), it was interpreted that any such effect of WAY-100,635 at D_4 receptors was inconsequential, and use of this compound was deemed appropriate for the current study. Likewise, it is important to note that, in the membranes of Chinese hamster ovary cells, WAY-100,635 has been shown to exhibit greater selectivity for human 5-HT_{1A} (pK_B = 9.47) than for human dopamine $D_{4.4}$ receptors (pK_B = 7.09) (pK_B being a negative logarithm of the equilibrium constant of, in this case, WAY-100,635) (Martel *et al.*, 2007), indicating a greater likelihood of 5-HT_{1A}-mediated effects to have been observed primarily. Indeed, post hoc analyses of the present study revealed that WAY-100,635 alone had no effect on locomotor activity, body temperature or stereotyped behaviour. In retrospect, this earlier study of Shortall *et al.* did not entail the combination of mephedrone with caffeine, and the relative enhancement of extracellular monoamine content following this combination might have rendered a differential receptor occupancy, though this does not appear to have notably altered resultant changes in locomotor hyperactivity, relative to rats pre-treated with vehicle.

Stereotyped behaviours were elicited by mephedrone, consistent with previous observations (Baumann *et al.*, 2012), and their severity was increased following co-administration of caffeine, consistent with the aetiology of the serotonin syndrome (Garrett & Sweeney, 2010; Mugele *et al.*, 2012) and observations in Chapter 2.

Although not verified with dialysate data in the present thesis, it would be expected that mephedrone and caffeine elicited increases in synaptic 5-HT, and that these increases would be substantial enough to precipitate the stereotyped behaviours observed presently. Based on review of the preclinical literature pertaining to the serotonin syndrome, it was hypothesised that these behaviours were elicited indirectly via 5-HT activation of postsynaptic 5-HT_{1A} receptors, and that the striatum played a critical role in mediating these behaviours. However, the present failure of WAY-100,635 to prevent these behaviours, when administered at a dose previously shown to be effective at preventing these behaviours in response to other serotonergic compounds, perhaps indicates the involvement of targets other than the postsynaptic 5-HT_{1A} receptors, or an insufficient level of occupancy at this receptor in the present study.

In this respect, it is not possible to rule out the potential involvement of pre-synaptic 5-HT_{1A} autoreceptors in the presently observed effects. These autoreceptors, somatodendritic and located on serotonergic neurons of the dorsal raphe nuclei (DRN) are implicated in negative regulation of the serotonergic system, whereby their activation by local 5-HT inhibits the firing and consequently also 5-HT release in forebrain projection areas (Innis & Aghajanian, 1987; Blier *et al.*, 1998; Liu *et al.*, 2005; Courtney & Ford, 2016). Stimulation of the DRN, for instance, has been shown to elicit increases in 5-HT release in dorsal striatum, globus pallidus and ventral hippocampus (McQuade & Sharp, 1997). Although there is evidence to suggest a role for both the dorsal and medial raphe nuclei in serotonergic innervation of the striatum (Behzadi, 2012), associated reductions in tryptophan hydroxylase activity of striatum (amongst other regions) were only associated with lesioning of the DRN, but not the median or lateral raphe nuclei (Geyer *et al.*, 1976), suggesting serotonergic projections from the DRN to striatum to be of particular importance to the presently observed effects of WAY-100,635. Although the dose of WAY-100,635 utilised here has previously been indicated to selectively inhibit post-synaptic 5-HT_{1A} receptors, as suggested by its prevention of behavioural changes related to this receptor subtype (Dupre *et al.*, 2011; Kawano *et al.*, 2015; Shortall *et al.*, 2016b), the present involvement of autoreceptors

cannot be excluded. For instance, the effect of WAY-100,635 on rapid eye movement (REM) sleep exhibited by adult rats appears to be contingent on administration route, whereby decreases and increases were observed following subcutaneous injection and microinfusion into the laterodorsal tegmental nucleus, respectively (Monti & Jantos, 2004), and indeed systemic co-administration of a dose of WAY-100,635 identical to that used in this chapter has previously been shown to prevent decreases in 5-HT efflux resultant of the 5-HT_{1A} agonist alnespirone in the frontal cortex of Wistar rats (Casanovas *et al.*, 1997). It is therefore possible that the systemic administration of WAY-100,635 utilised presently (rather than direct microinfusion into the striatum) might have resulted in antagonism of 5-HT_{1A} autoreceptors in the DRN, thereby disinhibiting 5-HT neuronal firing from neurons projecting from this region to other brain areas, causing a further increase in synaptic 5-HT content **(see Fig. 3.10)**. However, other suggestions for the failure of the 5-HT_{1A} receptor antagonist to prevent these behaviours may include activity of WAY-100,635 at D_4 receptors, and the differential receptor occupancy elicited by the combination of mephedrone and caffeine, relative to mephedrone alone, which may have enhanced 5-HT efflux to such an extent that WAY-100,635 was displaced, rendering the selected dose of this antagonist ineffective. The involvement of other receptors in this response can also not be ruled out. Hyperlocomotion, a component of the serotonin syndrome, has for instance been shown to be dose-dependently prevented as a response to MDPV administration as a result of CXC chemokine receptor type 4 (CXCR4) antagonism in rats (Oliver *et al.*, 2018). However, it is worth noting that there were no behavioural components of the serotonin syndrome thought to be mediated by activation of 5-HT_{2A} receptors observed, including wet dog shakes, head twitches or back muscle contractions in any rats which received mephedrone either alone or in combination with caffeine.

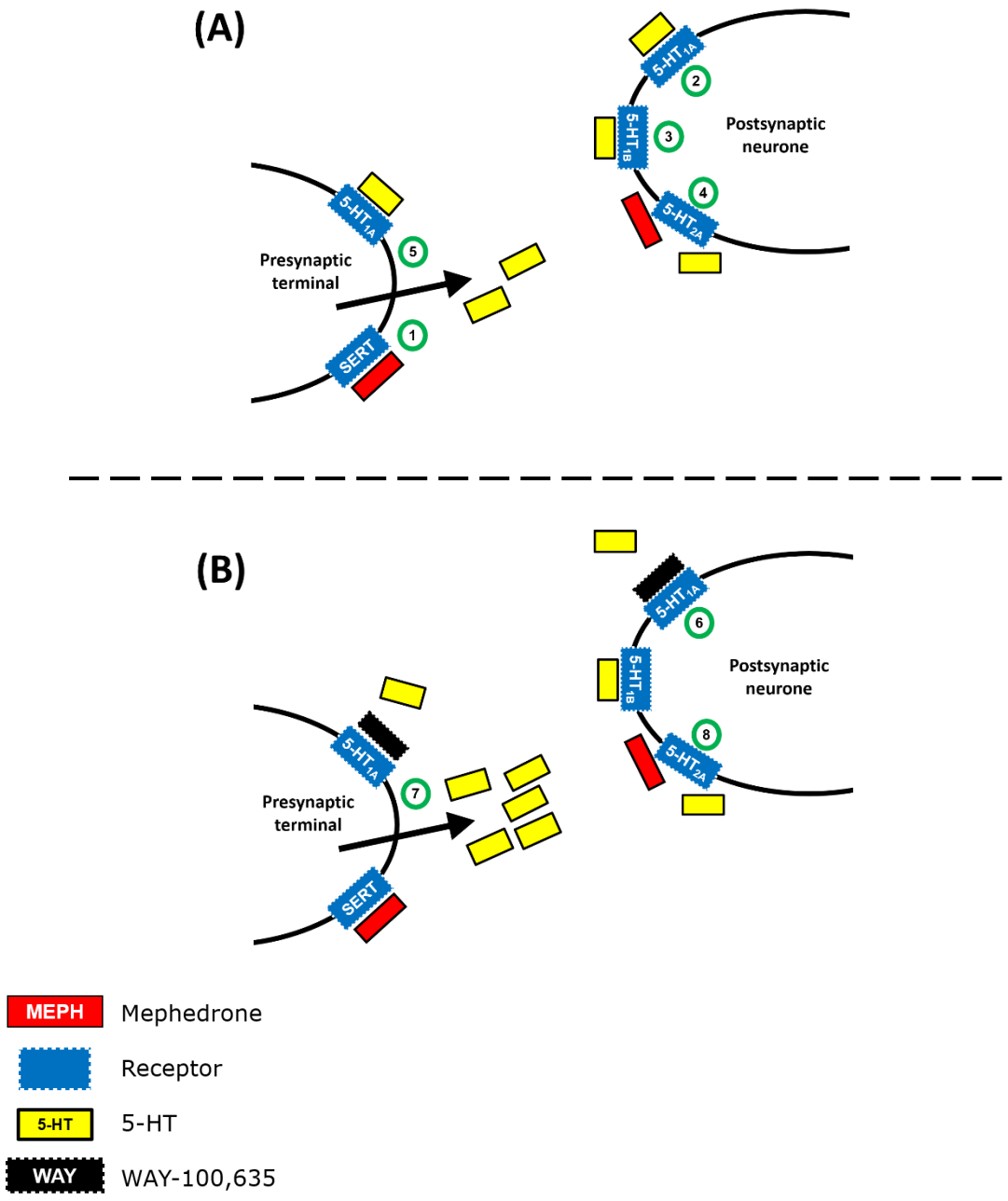

Figure 3.10 Diagram proposing the neurochemical mechanisms by which mephedrone and caffeine mediated the effects observed in the present chapter in the absence (A) and presence of WAY-100,635 (B). Mephedrone and caffeine elicit increases in synaptic levels of endogenous 5-HT via different mechanisms (mephedrone by its dual-mechanism as a transporter substrate and reuptake blocker (**1**); whilst caffeine causes downstream elevations via adenosine receptor antagonism); 5-HT binds to postsynaptic 5-HT_{1A} receptors, eliciting stereotyped behaviours resemblant of the serotonin syndrome (**2**); and also binds to 5-HT_{1B} receptors, effecting locomotor hyperactivity (**3**). Both mephedrone and synaptic 5-HT

exhibit high affinity binding at 5-HT_{2A} receptors, through which they are suggested to mediate the hyperthermic response to combination treatment **(4)**. Presynaptic 5-HT_{1A} receptors (or autoreceptors) are implicated in the regulation of synaptic 5-HT content by means of a negative feedback loop **(5)**. WAY-100,635 binds to postsynaptic 5-HT_{1A} receptors, which would be predicted to prevent the binding of 5-HT and the elicitation of stereotyped behaviours **(6)**. However, WAY-100,635 also binds to presynaptic 5-HT_{1A} receptors in the dorsal raphe nucleus, causing a disinhibition of serotonergic neurons projecting from this area, thereby causing increases in synaptic 5-HT elsewhere in the brain (including the striatum), which increases the likelihood of 5-HT binding to postsynaptic 5-HT receptors (including 5-HT_{1A}, which might explain the lack of effect of this antagonist of drug-induced stereotyped behaviours) **(7)**. These increases by WAY-100,635 also increases the likelihood of 5-HT binding at 5-HT_{2A} receptors and enhancing the hyperthermic effect elicited by mephedrone and caffeine co-administration **(8)**.

Collectively, these data on the effects of mephedrone and caffeine co-administration complement the findings of the previous study (Chapter 2), illustrating that additive effects following acute co-administration of mephedrone and caffeine parallel those resultant of caffeine's combination with MDMA (Vanattou-Saifoudine *et al.*, 2012), thereby suggesting the potential for exacerbation of adverse effects in recreational users. Unlike in the event of mephedrone-induced hypothermia (Shortall *et al.*, 2016b), the hyperthermia resultant of mephedrone and caffeine combination was not prevented by prior 5-HT_{1A} antagonism, and was in fact enhanced as a result of pre-administration of WAY-100,635. It is suggested that enhancement of the hyperthermic response, and the failure to prevent stereotyped behaviours, resulted from antagonism of WAY-100,635 on 5-HT_{1A} autoreceptors of the DRN, which disinhibited serotonergic neurons projecting from this area to other regions of the brain, rendering further increases in the bioavailability of synaptic 5-HT and further occupation of the 5-HT_{2A} receptors.

Chapter 4. General Discussion

4.1 Mephedrone in 2021

Between the rise of synthetic cathinones to popularity in Israel around 2007 (Green *et al.*, 2014; Nutt, 2020) to their prohibition throughout western countries at the beginning of the last decade (ACMD, 2010; EMCDDA, 2011; Haggin, 2012), mephedrone became established as a popular recreational drug of choice, a cheap alternative to and substitute for cocaine and MDMA (EMCDDA, 2010a; Winstock *et al.*, 2011a). In 2020, the European Monitoring Centre for Drugs and Drug Addiction reported that mephedrone use amongst 16 to 34 year olds in the UK had declined from 1.1% use within the previous year in 2014, to 0.0% in 2018 (EMCDDA, 2020b), consistent with a continuing decline in use since its 2010 peak (EMCDDA, 2017; Rice *et al.*, 2020; Vicknasingam *et al.*, 2020). Nonetheless, wastewater-based epidemiology (Castrignano *et al.*, 2018; Celma *et al.*, 2019; Brandeburová *et al.*, 2020), emergency presentations (EMCDDA, 2017; Ordak *et al.*, 2018), police seizures (Vicknasingam *et al.*, 2020), oral fluid samples (Bianchi *et al.*, 2019), surveys of adolescents (Guo *et al.*, 2019) and related fatalities (Hockenhull *et al.*, 2016; Palazzoli *et al.*, 2021) indicate that recreational use of mephedrone and synthetic cathinones persists, albeit at lesser frequency (Vicknasingam *et al.*, 2020). A 2018 seizure of 50 kilograms of the synthetic cathinone precursor 2-bromo-4-methylpropiophenone, as well as the discovery of mephedrone-producing laboratories in The Netherlands, Poland and Spain (EMCDDA, 2020b), support this proposition.

At the time of writing, there exists ten years' worth of preclinical data on the effects of mephedrone. The present body of work sought to increase understanding of the pharmacological mechanism underlying the effect of mephedrone and its interaction with caffeine, and thus expand on this literature, focusing in particular on the physiological effect of mephedrone on body temperature, as well as its behavioural effects on locomotor activity and stereotyped behaviour. Further lines of enquiry were the modification of these effects by caffeine, the role of downstream postsynaptic 5-HT_{1A} receptors in mediating these effects of drug combination, as well as probing the development of either sensitisation or tolerance to mephedrone's effects, long-term

changes in hippocampal microglial activation and potential deleterious effects on cognition.

4.2 Summary of findings

The psychoactive effects of mephedrone have been likened by recreational users to those of conventional/traditional stimulants, such as cocaine and MDMA. Indeed, the present thesis complements the findings of the broader preclinical literature, demonstrating that mephedrone elicits locomotor hyperactivity and changes in body temperature which are contingent on dose (whereby hypothermia and hyperthermia was observed following administration at 7.5 and 10.0 mg kg^{-1}, in Chapters 2 and 3, respectively) and dosing regimen.

To the best of the author's knowledge the contents of this thesis represent perhaps the first demonstration of the development of tolerance to the locomotor stimulating effect of mephedrone within one week of repeated dose administration – which may be indicative of the potential for this drug to be prone to abuse. The development of tolerance occurs when a human (or animal) no longer responds, or responds to a lesser extent, to a drug at a dose previously used, necessitating a higher dose to achieve the same effect. However, it is important to note that the reduction in locomotor activity presently observed on day eight, relative to day one, might be consequential of rats having become accustomed to the experimental apparatus (Chapter 2). In addition to this, it is believed to be the first illustration of caffeine's enhancement of mephedrone-induced stereotyped behaviours has been provided, highlighting a clinical issue of concern which may be experienced by recreational users co-ingesting these substances (i.e. the serotonin syndrome). Two further novel findings which have been described are the effects of repeated binge dose administration of caffeine with mephedrone on body temperature and locomotor hyperactivity. These findings showed the ability of caffeine to modify the effects of mephedrone in the rat, eliciting hyperthermia and hyperlocomotion which exceeded that observed following repeated dosing of mephedrone alone. These findings are of particular relevance given that

locomotor hyperactivity and hyperthermia are often symptoms of the serotonin syndrome, and their presence can provide medical professionals the opportunity to diagnose this condition in recreational users exhibiting them. This thesis also constitutes the first known examination of the effect of repeated binge dose administration of this drug combination in adolescent rats on measures of locomotor activity, recognition memory, aversion, sensorimotor gating, conditioned freezing and hippocampal microglial activation in adulthood, whereby no deleterious effects were observed. Finally, this marks the first known attempt to characterise the pharmacological mechanism by which the stereotyped behaviours observed in response to this drug combination are elicited, whereby systemic pre-administration of the 5-HT_{1A} antagonist WAY-100,635 failed to prevent stereotyped behaviours and in fact increased the hyperthermic response, perhaps through downstream effects of this ligand at 5-HT_{1A} autoreceptors elsewhere in the brain.

It is important to note that despite the absence of long-term effects of mephedrone and caffeine co-administration in the present thesis, this does not preclude the possibility of such effects being observed elsewhere. Only one chronic dosing regime was investigated, and this may not adequately mirror long-term patterns of multi-drug use, at increased ambient temperatures, in recreational settings. Despite best efforts to devise experiments which allow for translational relevance to human recreational users, preclinical studies are not conclusive, and the two studies outlined in this thesis are just two studies. Groups elsewhere have already and may continue to find the administration of mephedrone (with or without caffeine) to elicit deleterious effects on cognitive (Motbey *et al.*, 2012b; den Hollander *et al.*, 2013; Shortall *et al.*, 2013b; Lopez-Arnau *et al.*, 2015; Ciudad-Roberts *et al.*, 2016b) and biological measures, including dopaminergic and serotonergic deficits, as well as oxidative damage (Hadlock *et al.*, 2011; Motbey *et al.*, 2013; Lopez-Arnau *et al.*, 2015; Kaminska *et al.*, 2018). It is also worth noting that due to the unregulated nature of mephedrone (as well as other synthetic cathinones and illegal drugs in general), the accuracy of modelling the effects of products sold as “mephedrone” is impeded as a result of the numerous adulterants these products might contain, and that the findings of the

present thesis do not conclusively point to repeated binge dose administration of mephedrone with or without caffeine being inconsequential.

Despite endeavouring for a period of eight months to resolve them, technical issues with HPLC-ED laboratory equipment rendered analysis of the *in vivo* neurochemical correlates of these physiological effects impossible within the time frame of the present scholarship. It is also worth noting that the first experiment conducted for this thesis was delayed by four months as a result of the Controlled Drugs licence required to conduct this experiment not being renewed in time by the holder **(Appendix II)**.

4.3 Translational relevance

A fundamental consideration in the preclinical study of pharmacological substances is the utilisation of doses and dosing regimens pertinent to recreational use in humans. In satisfying this, pharmacokinetic and pharmacodynamic information both clinical and preclinical is necessary. Doses and dosing regimens of mephedrone and caffeine used in Chapter 2 were selected in order to mimic the practice of weekend use and re-dosing (Schifano *et al.*, 2011) observed in recreational mephedrone users. The doses employed for acute administration in Chapter 3 were selected based on the findings of the previous experiment and a separate pilot study which determined them suitable for eliciting measurable physiological changes, including stereotyped behaviours characteristic of the serotonin syndrome.

In human users, mephedrone is typically consumed orally or via insufflation at a dose ranging from 100.0 to 200.0 mg (for a user weighing 70 kg, this equates to approximately 1.5 to 3.0 mg kg^{-1}). The short duration of psychoactive augmentation however precipitates rapid re-dosing in users, in an effort to re-achieve the short-lasting high. Previous studies of mephedrone by this laboratory group and others have employed doses in a range similar to those used herein (7.5 to 10.0 mg kg^{-1}), observing measurable physiological changes which return to baseline within 1 h of

administration, analogous to the return to baseline cited in human users. It was important that the selected dose of mephedrone was high enough to elicit these changes, but sub-maximal so that any interaction effect of the co-administration of caffeine would be measurable. A previous study by our group examining the effect of caffeine's combination with mephedrone used 10.0 mg kg^{-1} to this effect (Shortall *et al.*, 2016a), allowing for the isolation and analysis of the effect of each compound. It was likewise important that the dose of caffeine be appropriate, as the co-use of this compound by recreational users of mephedrone might offer discernible changes in physiological and neurochemical effects. Nonetheless, despite rationalised choices of dose selection in preclinical models, the main limiting factors in the translational relevance of these selections resides in the inability to ascertain the exact doses ingested by recreational users, by virtue of illegal drugs being unregulated, and information on composition being unavailable. Likewise, no information is provided to users of any adulterants in the product they are consuming, each of which might exert their own unique or semi-unique pharmacological effects when combined with mephedrone.

It is frequently suggested that the translational relevance of preclinical rodent studies is limited, and whilst such studies are useful for the examination of pharmacological mechanisms of drug-induced effects, they are poor in terms of predicting the long-term consequences of such drugs. A good example in this respect is that of MDMA, which at certain doses and regimens in rats exerts neurotoxicity to serotonergic neurons as a result of peripheral metabolism, production of free radicals and induction of oxidative stress. In mice, however, this neurotoxic effect is exerted on dopaminergic neurons, whilst in humans, MDMA is metabolised much more slowly, and the plasma half-life is ten time longer, suggesting that the neurotoxic effects noted in rats following some dosing regimens might not result in a similar consequence in human users (Green *et al.*, 2012). Furthermore, when used for recreational purposes in man, the unpleasant side effects of MDMA would make it unlikely that high levels of MDMA which might produce neurotoxicity would be ingested, unlike in rodents where the dose is provided by the experimenter. Relative to MDMA, the area of preclinical study

into the effects of mephedrone remains relatively young, and more studies are warranted in order to better ascertain whether the effects described in this thesis and elsewhere are truly reflective of those experienced by human users. Going forward, a key consideration in this respect must be the employment of doses, regimens, and drug combinations reflective of the recreational experience of mephedrone use in society more generally.

The observation of tolerance toward the locomotor stimulating effect of mephedrone with repeated use is in keeping with the reports of tolerance and cravings described by recreational users. Importantly, this finding is consistent with clinical data illustrating the abuse potential of mephedrone, as well as its action to increase extracellular dopamine levels in preclinical models. Of course, the risk of abuse for a particular drug is elevated when this drug is taken either repeatedly in quick succession, or in combination with other drugs which cause further increases in dopamine release from ventral tegmental dopaminergic nerve terminals in the nucleus accumbens; eliciting the sensation of reward, initially in response to the drug experience itself, and subsequently in anticipation of this reward. This constitutes another rationale for the application of doses and dosing regimens with real world comparison to the human use of drugs of abuse. More generally, it is worth noting that, as described in Chapter 1, the modelling of drug abuse is more efficacious in rats than in mice, enhancing the translational relevance of this finding.

The stereotyped behaviours illustrated in this thesis are characteristic components of the serotonin syndrome, which may constitute a major clinical problem for users co-ingesting multiple drugs which each enhance serotonin release. Although mild cases of this syndrome can be treated by relatively minor measures which include discontinuation of the agents concerned and benzodiazepine or cyproheptadine administration, as well as observation of the patient for up to 24 hours, moderate and severe cases in which hyperthermia manifests as a symptom require further measures such as the administration of antipyretics. As suggested in this thesis, the

hyperthermic response to mephedrone and caffeine's combination is suggested to result from activation of 5-HT_{2A} receptors. Antagonists at this receptor, such as cyproheptadine, have been employed in the treatment of this symptom, with patients typically responding within one or two hours of initial administration (Graudins *et al.*, 1998). It was also proposed in this thesis that the stereotyped behaviours observed were consequential of activation of the 5-HT_{1A} receptors. Clinically, this receptor is also a target of interest, and compounds such as chlorpromazine, which functions as an antagonist at both 5-HT_{1A} and 5-HT_{2A} receptors (Wang *et al.*, 2016). The efficacy of these compounds to treat manifestations of the serotonin syndrome further verifies the relevance of the use of preclinical rat models in examining this condition in response to serotonergic drugs.

A less precarious study design in Chapter 3 would have allowed assessment of the involvement of an additional receptor, specifically 5-HT_{2A}. Such a design was prevented due to time constraint and financial limitations.

4.4 Future studies

Despite their prohibition between 2010 and 2012, the number of synthetic cathinones produced has rapidly increased. Although with time the popularity of any one drug may wane, it is prudent that elucidation of the pharmacological and physiological effects of currently available drugs continues; such is the case with mephedrone. Furthering the characterisation of mephedrone serves to aid future research to understand newly-marketed synthetic cathinones, enabling them to devise preclinical studies to assess their effects, make more reliable predictions of their effects in recreational users, and ultimately to advise health practitioners and policymakers. During the COVID-19 global pandemic, shortages in the heroin market have led to the employment of numerous substances as adulterants of heroin-based products, including synthetic opioids and synthetic cathinones (EMCDDA, 2020a). Recent history illustrates that when faced with shortages in the heroin market, recreational users have replaced traditional drugs with synthetic cathinones, such as for heroin in

Hungary (Tarján *et al.*, 2017) and cocaine in Slovenia (Sande, 2016). A recent study in Italy also indicates that mephedrone continues to be employed as an adulterant of products sold as MDMA (Fregonese *et al.*, 2021).

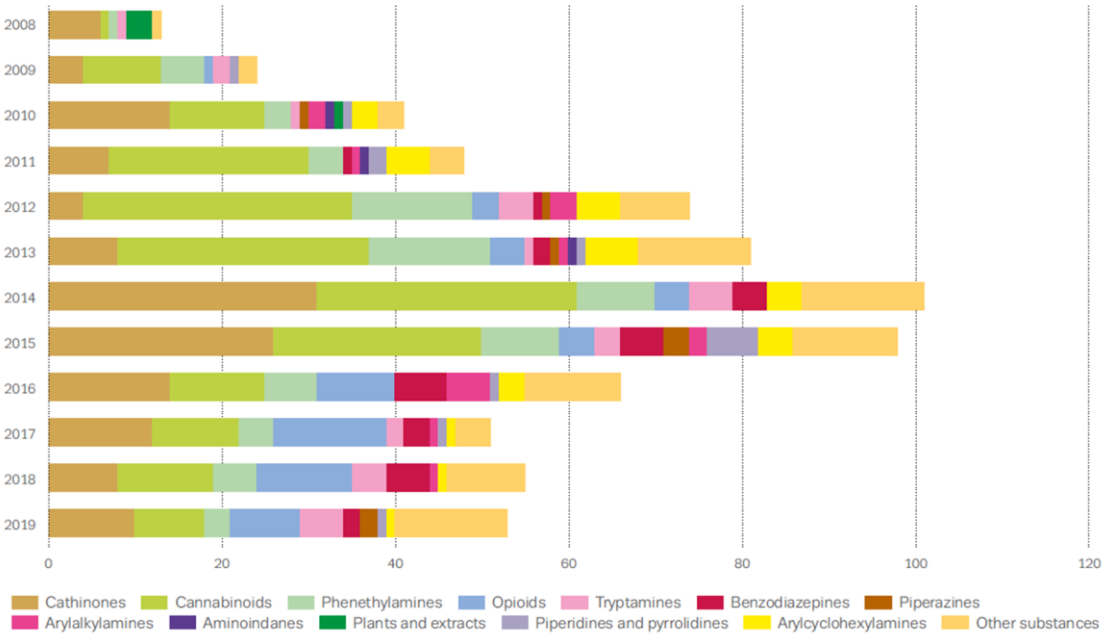

Figure 4.1 The numbers and types of novel psychoactive substances reported to the EU Early Warning System for the first time, from 2008 to 2019. The number of newly-reported synthetic cathinones peaked in 2014, but new synthetic cathinones have remained in production yearly until at least 2019. Figure taken from EMCDDA (2020b).

Although the neurotoxic potential of mephedrone remains inconclusive at the time of writing, it has been shown to enhance the neurotoxic effect of amphetamines (Angoa-Perez *et al.*, 2013) and induce pro-oxidative effects when combined with nicotine (Budzynska *et al.*, 2015) or alcohol (Ciudad-Roberts *et al.*, 2016b). Polydrug use involving synthetic cathinones therefore remains a cause for concern, for two reasons in particular. The first stems from data recently obtained by the European Syringe Collection and Analysis Project Enterprise (ESCAPE) network, indicating the presence of synthetic cathinones in used syringes in Helsinki, Budapest and Paris, the latter two for which synthetic cathinones accounted for 55 and 67% of syringes, respectively

(EMCDDA, 2020b). The second cause for concern pertains to the introduction of more potent derivatives of catinone and mephedrone to market, such as *N*-ethylhexedrone – structurally similar to α-PHP (a longer chain homologue of α-PVP) – which has recently been shown, at 100.0 μM, to induce microglial activation in human nerve cell lines (de Mello-Sampayo *et al.*, 2020). It is of utmost importance then that the existing sythetic cathinones, including mephedrone, be understood in greater detail, such that their effects and the effects of future chemically-related compounds be better anticipated in human users, and that efficacious treatment of resultant adverse effects can be devised.

A recent study on the facilitation of intracranial self-stimulation by MDPV observed that this effect could be inhibited by antagonism of the P2X7 receptor (Gentile *et al.*, 2019), indicating a role for P2X7 in the rewarding effects of this synthetic cathinone, though it must be noted that this is typically associated with activation at sufficiently high levels of ATP, rendering this possibility unlikely following the dosing regimens described presently. Nonetheless, P2X7 receptors – which are expressed in microglia of both rat (Yu *et al.*, 2008) and mouse (Chessell *et al.*, 1997) – mediate the release of proinflammatory cytokines, such as interleukins 1 beta (IL-1β) and 6 (IL-6), tumor necrosis factor alpha (TNF-α) and reactive oxygen species (ROS) upon activation in mice (Fernandes *et al.*, 2016; He *et al.*, 2017). Future investigation of mephedrone's pharmacological profile may benefit from probing a potential role of this receptor, antagonism of which has also been shown to reduce amphetamine-induced hyperactivity in both rats (Bhattacharya *et al.*, 2013) and mice (Csölle *et al.*, 2013; Gubert *et al.*, 2016). *In vivo*, such a study might include systemic administration of an antagonist with both high bioavailability and brain penetration, such as A428079 (Nelson *et al.*, 2006), and observing any resultant effect on the capacity of mephedrone to effect locomotor activation, facilitation of reward, conditioned place preference, increases in extracellular dopamine levels and/or cytokine release.

In terms of investigating the intrinsic rewarding properties of mephedrone, the measurement of neuronal activation following drug administration can be conducted through the use of established markers, such as the c-Fos transcription factor (Harlan & Garcia, 1998; Kovács, 2008). Previous work by our group determined that as well as increasing body temperature and locomotor activity, cathinone effected significant increases in c-Fos-immunopositive cells in both striatum and suprachiasmatic nucleus of Siberian hamsters (Jones *et al.*, 2014). Similar increases in locomotor activity and c-Fos expression in rat cortex, dorsal and ventral striatum, ventral tegmental area and supraoptic nucleus, have been observed in response to mephedrone (Motbey *et al.*, 2012a), with the activation of the cortical regions here being akin to that observed following MDMA and methamphetamine, and perhaps being indicative of the arousing and highly rewarding effects of the stimulant (Stephenson *et al.*, 1999). Since completion of the laboratory work associated with this PhD thesis, a follow up line of work within the laboratory group has involved investigation of c-Fos expression in brain regional samples from the animals described in Chapter 3. The c-Fos immunohistochemistry was the focus of a separate MRes project and is beyond the scope of the current PhD, although a joint conference abstract on the behavioural and immunohistochemical data has been submitted for presentation at the 2021 British Association of Psychopharmacology online meeting (O'Hara *et al.*, 2021). In summary, mephedrone was found to have increased c-Fos expression in NAcc core, whilst the combination of mephedrone with caffeine precipitated increases in both NAcc core and shell, as well as ventral striatum. The pre-administration of WAY-100,635 had no effect on any of these measures. Another marker which may be of use in this respect is BDNF, the presence of which is increased by psychostimulants (Graham *et al.*, 2007). Indeed, the expression of BDNF appears to underpin the developmental expression of D_3 receptors (Guillin *et al.*, 2001), which in turn are crucial for mediating the rewarding effects of drugs including methamphetamine (Higley *et al.*, 2011a; Higley *et al.*, 2011b).

In a separate but contextually important vein, whilst reviewing pre-existing literature for this thesis, it became apparent that a large proportion of mephedrone-related

publications pertained to the co-use of the drug with GHB/GBL and/or methamphetamine in recreational users, in the practice of “chemsex”. Of the potential combinations in this practice, only the effects of mephedrone with methamphetamine have been examined in a preclinical model, whereby mephedrone potentiated methamphetamine-induced depletions of striatal DA, DAT and TH (Angoa-Perez *et al.*, 2013). At the time of writing, no preclinical data are available on the interaction effects of mephedrone or methamphetamine with GHB and/or GBL. From a public health perspective, it is vital that any such interaction effects be elucidated as a matter of priority.

Given it is now over a decade since the first documented recreational use of mephedrone as a novel psychoactive substance, data concerning the long-term effects of the drug could perhaps be obtained from recreational users active during the early years of this period. Such data could entail measuring cognitive performance via testing, as well as physiological measurement of the function of major organs, such as the heart, in order to probe any lasting consequences of recreational use. This may prove of importance, as it has previously been indicated elsewhere that issues relating to cardiovascular health, such as valvular heart disease, may be elicited as a long-term effect of the use of stimulants such as MDMA (Cosyns *et al.*, 2013).

Finally, a long-standing issue arising from public health concerns and the prohibition of drugs has been an inability to critically assess their potential medicinal and/or therapeutic value. A substantial body of work has materialised in recent years which indicates the potential for numerous compounds, which remain illegal in much of the world, to be applied – often in adjunct with conventional therapeutic practices – to human users. Such examples include the use of: psilocybin (a naturally occurring psychedelic and the chief psychoactive component of “magic mushrooms”) in the treatment of anxiety and depression (Griffiths *et al.*, 2016; Patra, 2016; Goldberg *et al.*, 2020); MDMA in the treatment of generalised anxiety disorder and post-traumatic stress disorder (Sessa, 2017; De Gregorio *et al.*, 2021); and cannabis in the treatment

of various medical conditions, such as those which include pain as a symptom (Blake *et al.*, 2017; Ebbert *et al.*, 2018).

4.5 Conclusion

The present body of work has illustrated the potential of mephedrone to elicit behaviours resemblant of components of the serotonin syndrome in rats, following both repeated administration and co-administration of caffeine; two features reflective of recreational use in human mephedrone users. Exhibition of this syndrome can prove dangerous and indeed lethal in recreational users, and its elucidation therefore constitutes a valuable contribution to the understanding of recreational use of mephedrone and structurally-related derivatives.

Aside from complementing the existing literature in terms of mephedrone's effects on body temperature and locomotor activity, this thesis also determined that tolerance was developed toward the locomotor-stimulating effect of mephedrone within a week of repeated binge dose administration. This finding is indicative of mephedrone's abuse liability, and is consistent with both preclinical data of other synthetic cathinones and reports of recreational users.

The pharmacological profiles of co-ingested drugs are important in determining the effect of their interaction. Although it was not possible to verify the hypothesised pharmacological mechanism by which combination treatment of mephedrone and caffeine effected stereotyped behaviours (i.e. 5-HT activation of postsynaptic 5-HT_{1A} receptors), this might allow future studies to refine their scope in determining this mechanism, be it through alternative means of antagonist administration, or in seeking alternative pharmacological targets.

Although the recreational use of mephedrone has dwindled across the last decade, structurally-related derivatives continue to populate the market. Preclinically, improved understanding of the pharmacological, physiological, behavioural, cognitive and neurochemical effects of current synthetic cathinones serves only to strengthen the ability of clinical professionals, healthcare professionals and policymakers in making evidence-based decisions which positively impact the lives of those who might experience adverse effects in response to these structural derivatives. Despite the observation of no lasting effects of mephedrone alone or in combination with caffeine presently, this does not preclude the manifestation of such effects following different dosing regimens, or in response to the combination of mephedrone-related structural derivatives and other compounds. The rapid development of potentially dangerous synthetic cathinones necessitates a rapid development in our understanding; as does any remote possibility that these compounds might be of some therapeutic or medicinal value in years to come.

Appendix I HPLC troubleshooting

As described in Section **3.2.8**, the author had intended to analyse microdialysis samples using an established HPLC-ED method. In brief, chromatography apparatus and conditions comprised were to be injected on to a Luna® C18 3 µm 100 Å, LC Column (Phenomenex) using a Perkin Elmer AS200 autosampler (Antec Leyden; The Netherlands). An initial mobile phase composition 20.0 mM potassium dihydrogen phosphate (KH_2PO_4), 20.0 mM sodium acetate (NaAc), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.15 mM octanesulfonic acid (OSA) and 4.0% methanol (pH 3.89) would be circulated throughout the system at a flow rate of 0.05 ml min^{-1} via and Ultimate 3000 pump (Dionex) at a constant pressure of *circa* 1000 psi. Analytes were to be detected using an Antec VT-03 cell with a glassy carbon 2.0 mm working electrode at +0.59 V with an *in situ* Ag/AgCl ISAAC reference electrode.

However, it was not possible to detect five separate peaks corresponding to the monoamines 5-HT and dopamine and their key metabolites 5-HIAA, DOPAC and HVA with this composition, and those that were detected had very short retention times suggesting remaining analytes were either co-eluting and/or obscured within the solvent front.

Over a period of eight months (which was disrupted by five months lab closure due to regulations implemented in response to the COVID-19 pandemic) numerous alterations were made, initially to the mobile phase composition (to resolve issues of co-elution) and later to the pump (to address some technical issues) and cell (to address poor signal to noise ratio). These steps and the outcome are detailed in **Table A.1**, whilst representative chromatograms obtained throughout this troubleshooting phase are depicted in **Fig. A.1**. However, it ultimately proved not possible in terms of financial costs or time to resolve the issues to obtain sufficient sensitivity and separation required to detect 5-HT and dopamine in the 576 stored microdialysis samples.

Table A.1 Key troubleshooting changes made to HPLC-ED apparatus in order to achieve optimal parameters for the measurement of dopamine, 5-HT and their major metabolites in striatal dialysates.

Date	Modification	Outcome(s)
12/03/20	Decreased MeOH (8.0 → 4.0%)	DOPAC obscured by solvent front; Retention time of dopamine remained early
13/03/20 – 24/08/20	Lab work suspended due to COVID-19 restrictions; HPLC pump flushed with 20% MeOH, and apparatus shut down.	
27/08/20	Increased OSA to delay retention of amine components (0.15 → 0.17 M)	Co-elution of peaks
02/09/20	Increased OSA (0.17 → 0.20 M); Decreased pH (3.89 → 3.60)	Five distinct peaks observed in pooled standard; Large solvent front in spare basal dialysate obscured DOPAC and dopamine peaks; Visible peaks lower in amplitude in spare basal dialysates than pooled standards.
02/09/20	Increased injection volume (10.0 → 15.0 µL)	5-HT peak low in amplitude and broad in width; DOPAC and dopamine peaks co-eluting, largely obscured by solvent front.
07/09/20	Decreased MeOH (4.0 → 3.0%)	Corresponding peaks observed in pooled and individual standards; Large solvent front following injection in spare basal dialysate, obscuring DOPAC peak;

		Dopamine and 5-HT peaks absent in spare basal dialysate.
12/09/20	HPLC pump piston seals began leaking so were replaced. This involved a seven week delay while required supplier added to new University purchasing system.	
04/11/20	Increased pH (3.60 → 3.89); Decreased cell voltage (+0.75 → +0.59 V)	Only two peaks detected in pooled standards; 5-HT peak observed only following ten-fold higher concentration (150 pmol); Not possible to detect dopamine, DOPAC or HVA peaks.
05/11/20	Decreased cell voltage (+0.59 → +0.46 V)	No dopamine peak detected in individual or pooled standard; Smaller 5-HT peak, relative to following higher voltages.
06/11/20	Increased cell voltage (+0.46 → +0.75 V); Decreased MeOH (3.89 → 3.60)	Five distinct peaks in pooled standard at concentration of 7.5 pmol; However, comparison of chromatograms with those obtained before HPLC pump broke revealed these weren't as sensitive as settings achieved earlier.
03/03/21	New guard column (OPTI-GUARD ® 1.0 mm C18) installed.	
05/03/21	N/A	Five distinct peaks in pooled and individual standards, at concentration of 1.5 pmol; Substantial amount of baseline noise.
29/03/21	New working electrode block installed	
30/03/21	N/A	Four distinct peaks in pooled and individual standards, at concentration of 1.5 pmol;

		Baseline noise appears minimised.
31/03/21	Increased pH (to 3.73)	Co-elution of dopamine with 5-HIAA.
01/04/21	Increased pH (to 3.90)	Peaks for amine components shifted to later retention times; Distinct peak for dopamine absent, suggesting further change in pH required.
07/04/21	Decreased pH (to 3.50)	Partial separation of peaks for dopamine and 5-HIAA.
08/04/21	Decreased pH (to 3.30)	Further separation of peaks for dopamine and 5-HIAA.
09/04/21	Decreased pH (to 3.10)	Further separation of peaks for dopamine and 5-HIAA; No peaks for dopamine or 5-HT observed following injection of basal dialysate spare.
13/04/21	Baseline noise became more substantial, suspected to be consequential of issues with HPLC pump. This was confirmed by decreasing flow rate from 0.05 to 0.04 ml min^{-1}, which elongated the issue, and a further change of piston seals failed to resolve this issue. A final decision (from the point of view of completing this thesis in a timely manner now that the period of the stipend for living expenses had terminated) was made that online advice would be sought from Antec, Inc. and most likely an on-site service of the system would be required, entailing further expenditure on the pump. Financial outlay at this point had already exceeded the consumable funding provided for this PhD project, so it is not feasible for this on-site service to occur at present.	

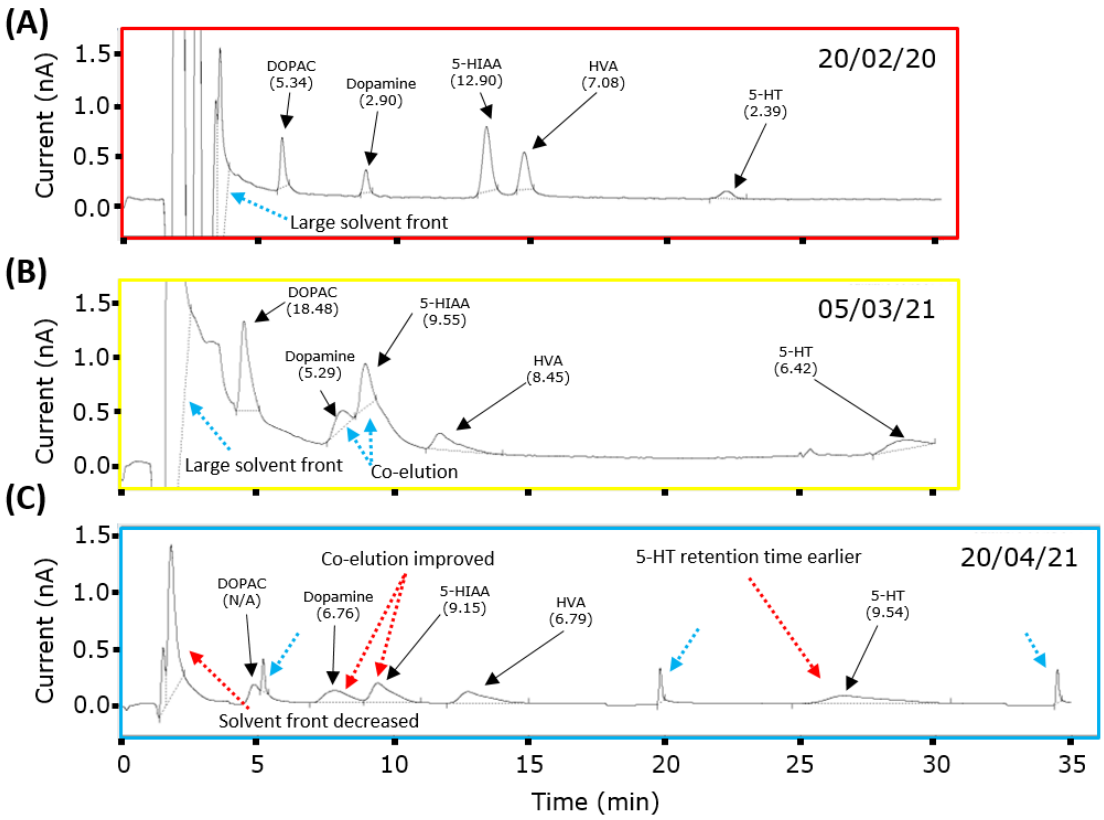

Figure A.1 Chromatograms depicting peaks detected corresponding to dopamine, 5-HT and their major metabolites. With area under curve (nA.s), as measured following injection of pooled standards in the early **(A)**, middle **(B)**, and final **(C)** stages of optimisation. Dotted blue lines illustrate some issues with chromatogram at time of observation: in panels **(A)** and **(B)**, a large solvent front; in panel **(B)**, co-elution of dopamine and 5-HIAA; in panel **(C)**, baseline noise approximately every 15 minutes. Dotted red lines depict resolutions to some of the issues encountered during troubleshooting: in panel **(C)**, these include the decreases in the amplitude of the solvent front (as a result of installation of a new electrode block) shifting of 5-HT retention time earlier (due to reduction in pH), and the reduction of co-elution of dopamine with 5-HIAA (by increasing the ion pair).

In sum, increasing the ion pair in the mobile phase resolved the co-elution, reducing the methanol concentration rendered separation of analyte peaks from the solvent front, and the installation of a new electrode block increased sensitivity.

Despite all these modifications, there was still insufficient sensitivity to enable basal levels of dopamine and 5-HT to be measured in microdialysis samples. Some microdialysis samples were provided to RenaSci, who were able to confirm that the expected levels of dopamine and 5-HT were present in the samples, and that the collection procedure had worked, ruling out the possibility of issues from data collection.

This thesis should have involved three years full time lab work plus a one year thesis pending period. It was not possible to conduct practical work for over five of the final months of this planned period due to restrictions enacted by the University of Nottingham and UK government in response to the COVID-19 pandemic. However, once relevant restrictions were lifted, lab access and full-time work was resumed and conducted for over seven months, thus exceeding the planned period of study by more than six months.

Appendix II Gantt chart of experimental work conducted

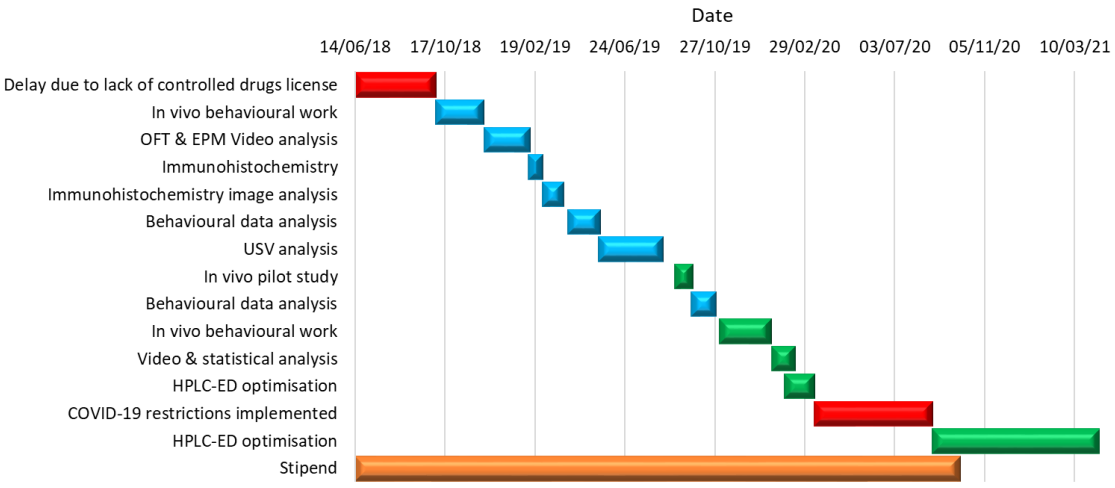

Figure A.2 Gantt chart depicting the dates of experimental work conducted throughout this research degree programme. Experimental work conducted for experiments one (blue bars) and two (green bars), as well as disruptions caused to experimental work (red bars).

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