THE EFFECT OF MEPHEDRONE AND CAFFEINE, AND THE INFLUENCE OF WAY-100635 ON C-FOS EXPRESSION IN THE RAT BRAIN

LUCY ADAM

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Abstract

Mephedrone is a commonly abused drug, with recreational users comparing its effects to MDMA. Polydrug use of mephedrone has been widely documented and forensic analysis has identified caffeine as key drug that is co-consumed. The current study aimed to identify the changes in c-fos expression, a commonly used marker in research to determine neuronal stimulation in response to mephedrone and caffeine administration, and the effect that inhibition of the 5-HT_{1A} receptor has on this expression.

48 male Lister Hooded rats received i.p. vehicle (saline 1mL/kg) or WAY-100635 (0.5mg/kg) then 30min later vehicle, caffeine (10mg/kg), mephedrone (10mg/kg) or caffeine plus mephedrone (10mg/kg each). Animals were killed using Euthatal and brains were removed to determine c-Fos expression. (**This *in vivo* work was performed by a previous PhD student).** Free-floating sections cut coronally at 60μm, and immunohistochemistry was performed.

Mephedrone displayed a main effect in the dorsal striatum, pre-limbic cortex, the nucleus accumbens core and shell. In the shell, the post-hoc tests showed a significant difference between the vehicle + vehicle treatment group and the vehicle + mephedrone treatment groups. When combined with caffeine, there was a main effect observed in the dorsal striatum and the hypothalamus, however no significance was seen between groups using Tukey's post-hoc tests in either of these regions. In contrast, the post-hoc tests showed a significant increase between the vehicle + vehicle treatment group and the vehicle + mephedrone & caffeine treatment groups observed in the nucleus accumbens shell, core, ventral striatum and pre-limbic cortex. There was no effect of WAY-100635 in any experimental condition.

The results suggest that caffeine displays an additive effect when combined with mephedrone, suggesting it may exacerbate mephedrone induced serotonergic syndrome. Unlike mephedrone alone, the combination of caffeine and mephedrone does not appear to be affected by 5-HT_{1A} receptor antagonism and more work should be completed to investigate the downstream involvement of 5-HT_{1B}, 5-HT_{2A} and dopaminergic receptors.

Publications

Abstracts

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.

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Covid impact statement

Due to the impact of COVID-19, some aspects of the study were not completed as planned. Fluorescence immunohistochemistry to identify co-localisation of the c-fos^{+} cells would have been preferred; however, it was decided to not be carried out due to the instability of fluorescent staining could result in loss of signal if isolation, or long-term laboratory closure would be required because of the pandemic. Therefore, a more permanent DAB staining was used as this could last indefinitely before the sections were counted. More regions of interest would have also been chosen, such as the raphe nucleus to identify serotonergic input, however due to periods of isolation and five months of laboratory closure, it was not possible to complete further analysis during the project.

Abbreviations

5-HIAA - 5-hydroxyindoleacetic acid

5-HT – Serotonin

AC – Adenylate cyclase

AMP – Adenosine monophosphate

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic

ATP – Adenosine 5'-triphosphate

BBB -Blood Brain Barrier

CNS – Central Nervous system

COMT - Catechol-O-methyltransferase

Cp – Canduate putamen

CPP – Conditioned place preference

CREB - cAMP response binding protein

Cmax – Maximum plasma concentration

DA – Dopamine

DAB – 3,3'-diaminobenzidine

DAG – Diacylglycerol

DAT - Dopamine transporter

DOPAC - 4-dihydroxyphenylacetic acid

DPX - Dibutylphthalate polystyrene xylene

EPM – Elevated plus maze

EPSC - Excitatory postsynaptic current

Fig. – Figure

fMRI - functional magnetic resonance imaging

GABA – Gamma-aminobutyric acid

GBL – Gamma-butyrolactone

GFAP - Glial fibrillary acidic protein

GHB – Gamma-hydroxybutyrate

GIRK - G-protein-coupled inward rectifying potassium channel

GPCR – G-protein coupled receptor

HPLC - High Performance Liquid Chromatography

HVA - Homovanillic acid

ICSS – Intracranial self-stimulation

IP3 – 1,4,5-trisphosphate

K_e – Elimination rate constant

L-dopa - l-3,4-dihydroxyphenylalanine

LC - Locus Coeruleus

LMA – Locomotor activity

LTD – Long Term Depression

LTP – Long Term Potentiation

MDMA - 3,4-Methylenedioxymethamphetamine

MDPV - 4-methylenedioxypyrovalerone

MOA - monoamine oxidase

Methylone - 3,4-methylenedioxy-N-methylcathinone

NDMA – N-methyl-D-aspartate

NE - Noradrenaline

NET – Noradrenergic transporter

NO – Nitric oxide

NPS – Novel psychoactive substance

Nac – Nucleus accumbens

NacC - Nucleus accumbens core

NacS - Nucleus accumbens shell

OCT - Optimal cutting temperature

PBS - In phosphate buffered saline

PFC -Prefrontal cortex

PIP2 - Phosphatidylinositol 4,5-bisphosphate

PKA – Phosphokinase A

PLC – Phospholipase C

POA - Preoptic area of anterior hypothalamus

PrL – Prelimbic cortex

ROS - Reactive oxygen species

SEM – Standard error of the mean

SERT – Serotonin transporter

$T_{1/2}$ – Half life

TH – Tyrosine hydroxylase

Tmax – Fast absorption time

VMAT - Vesicular monoamine transporter 2

VTA – Ventral tegmental area

WAY-100635 - N-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-N-(2-pyridyl)-cyclohexanecarboxamide maleate

cAMP - 3'-5' cyclic adenosine monophosphate

fMRI - Magnetic renosance imaging

i.p. – Intraperitoneal

i.v. – Intravenous

iNOS - inducible nitric oxide synthase

mPFC – Medial prefrontal cortex

mg kg-1 – Milligrams per kilogram of body weight

s.c. – Subcutaneous

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1. Introduction

1.1 Mephedrone

Cathinone's are monoamine alkaloids derived from the khat plant (Catha edulis) that are used recreationally by users who want to induce feelings of euphoria, decrease inhibition, enhance sensory capability and experience increased energy, libido and empathy to others (Singh and Juneja, 2019). The leaves of the khat plant contain the naturally occurring beta-ketone which, when chewed, induces the psychoactive effects similar to those seen with amphetamine (Prosser and Lelson, 2012), which can be attributed to their similarities in chemical structure (seen in Figure 1). Khat is native to East Africa and the Arabian Peninsula and historical evidence suggests that khat has been used since the 13th century in Ethiopia (El-Menyar et al, 2015). It was not until the mid-20th century that it was exported across the globe, to nations such as the United States and the United Kingdom (Oliver et al, 2018). Patient reports show that it is still commonly used with 200 million people worldwide chewing khat for its stimulant effects (El-Menyar et al, 2015), there being notably high prevalence in Yemen, Kenya, Ethiopia, Eritrea, Somalia, and Saudi Arabia (Al-Maweri, Warnakulasuriya and Samran, 2017).

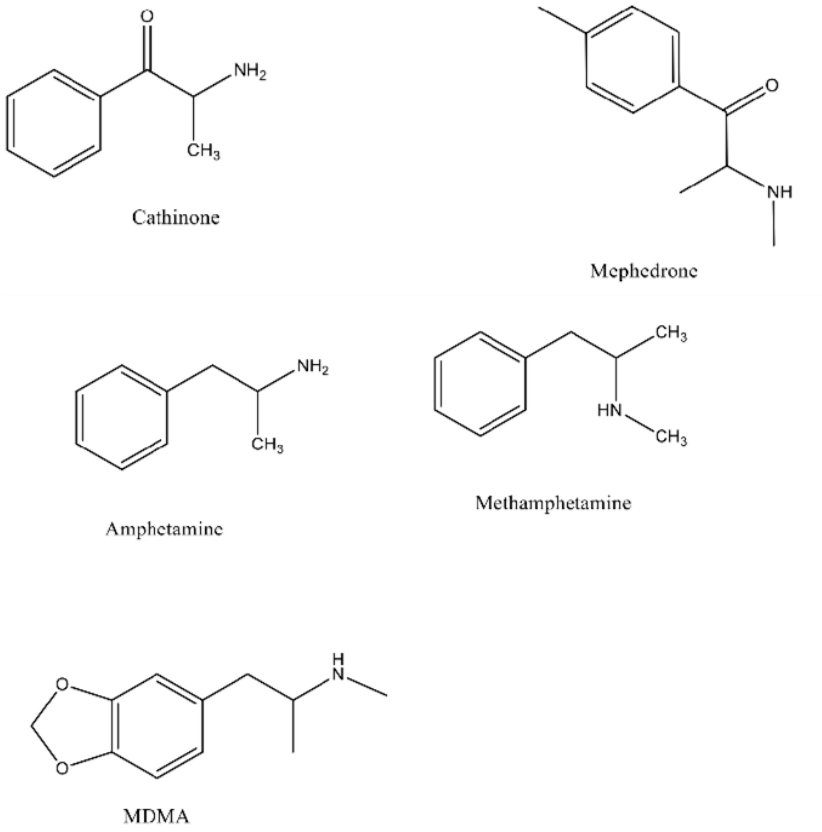

Figure 1. Structural similarities between related compounds

Molecular structures of MDMA, mephedrone, cathinone and amphetamine (Pantano et al, 2016)

Cathinone's are phenethylamine derivatives, with the defining property of the presence of the beta-ketone in their chemical structure (Papaseit et al, 2016). In the 1970s, the psychoactive compound, cathinone, was isolated from the khat plant by The United Nations Narcotics Laboratory (Oliver et al, 2018), and the structure used as a chemical template for the creation of similar drugs with psychoactive effects (UNODC, 2015). These illicit synthetic cathinone's are termed "designer drugs" which are often in the form of crystalline powders, although sometimes found in the form of pills and capsules (Milena et al, 2018). Commonly produced in China or other Asian countries (Hägele, Hubner and Schmid, 2019) but packaged in Europe (EMCDDA, 2011) synthetic cathinone's often labelled with "not for human consumption, "plant food" and/or "bath salts" to bypass legal enforcement, with Europe and the UK's most common being 4-methylmethcathinone (mephedrone).

Acute subjective effects	Acute physiological effects
Increased Libido	Increased heart rate
Jaw clenching	Increased systolic blood pressure
Anxiety	Increased diastolic blood pressure
Euphoria	Increased pupil diameter
Body sweats	
Chest pain	
Increased heart rate	

Table 1. Summary of the acute effects induced by mephedrone
Data abstained by Papaseit et al (2020)

1.1.1 Pharmacokinetics

Mephedrone is most commonly self-administered via insufflation, however oral, and even less commonly, i.v., routes are also used (Baumann, Partilla and Lehner, 2013). Oral doses have been reported to range between 75 and 100mg compared with 20 - 80mg for insufflation (Erowid, 2015). The higher dosage seen with oral administration coincides with the low bioavailability of mephedrone when administered in this form, compared with the high bioavailability when taken via insufflation (Martínez-Clemente et al, 2013), which justifies the reason recreational users prefer to administer via the latter.

The pharmacokinetics of mephedrone reflect the administration time and dosage. Any compound with brain/plasma ratio higher than 1 is expected to easily cross the blood brain barrier (BBB) Mephedrone has a brain/plasma ratio of 1.85, thus freely crosses the BBB. (Martínez-Clement et al, 2013). This is in agreement with the fast absorption time (T*max*) seen in both rats and humans (Czerwinska et al, 2020). In humans, a dose of 150mg of mephedrone ingested orally produces a T*max* of 1.2 hours, $t_{1/2}$ of 2.2 hours, C*max* of 179.0 ng/mL, and has a K_e of 0.32 h-1 (Olesti et al, 2017). In rats, an oral dose of 30mg/kg has a T*max* of 0.93h, a C*max* of 331µg/kg, and a $T_{1/2}$ of 2.15 hours (Martínez-Clement et al, 2013). Compared with MDMA, mephedrone has a relatively short half-life (Papaseit et al, 2020), which explains the reason why recreational users redoes more often, in a similar manner to cocaine (EMCDD, 2011). The clear differences in pharmacokinetics compared with other, similar drugs of abuse (summarized in Table 2) highlights the importance of identifying the full profile of a substance, and not making presumptions based on structurally similar compounds.

Pharmacokinetic Parameter	**Mephedrone (150mg)**	**Mephedrone (200mg)**	**Cocaine (200mg)**	**Methamphetamine (20mg)**	**MDMA (100mg)**
Absorption (T*max*) (h)	1.2	1.25	1.3	7.5	2.3
Half-life ($t_{1/2}$) (h)	2.2	2.15	1.3	11.1 h	9.0
Maximum plasma concentration (C*max*) (Ng/mL)	179.0	134.6	268	32.4	222.5
Elimination rate constant (Ke) (h^{-1})	0.32	0.33	x	x	2.7

Table 2. Pharmacokinetic comparison of mephedrone to other drugs of abuse in humans

All data is represented as the mean. X implies no data. Compared with cocaine, methamphetamine and MDMA, mephedrone has a shorter absorption time and a larger maximum plasma concentration. Compared with cocaine, mephedrone has a longer half-life, but much shorter compared with methamphetamine and MDMA.
(Data derived from; [1]Olesti et al (2017); [2]Schephers et al, (2002);[3]de la Torre et al (2004); Coe et al (2018); Papaseit et al (2015)).

1.1.2 Site of action

It is well documented that mephedrone is non-selective, acting on the DAT (dopamine transporter), SERT (serotonin, also known as 5-HT, transporter) and noradrenergic transporter (NET), (Hadlock et al, 2011; Baumann et al. 2011; Lopez-Arnau et al, 2015; Baumann et al, 2013). Mephedrone displays the highest affinity for SERT, followed by DAT and then by NET, respectively (summarized in Table 3). Previous studies have indicated that by binding to these transporters, mephedrone blocks the reuptake of their respective monoamines. This results in an increase in concentration of the monoamines, which produces the effects observed in users (Pifl, Reither and Hornykiewicx, 2015, Martinez-Clemente et al, 2011). This is a similar mechanism by which other drugs of abuse produce their effects (i.e., influencing the concentration of the central monoamines; summarised in table 3)

Receptor:	Mephedrone	MDMA	Amphetamine
DAT	3.4 ± 0.8	6.5 ± 2.5	5.68 ± 3.8
SERT	>30	13.3 ± 0.6	>25
NAT	>25	30.5 ± 8.0	1.00 ± 0.6
5-HT $_{1A}$	>20	12.2 ± 0.8	6.74 ± 1.38
5-HT $_{2A}$	2.1 ± 0.7	7.8 ± 2.4	>13
D_1	>13.6	>13.6	>13.6
D_2	>30	25.2 ± 12	>30
α_{1A}	3.48 ± 2.2	>6	>6
α_{2A}	11.0 ± 5.0	15.0 ± 10	2.8 ± 0.8

Table 3. Binding affinities for monoamine transporters and receptors

Data obtained from Simmeler et al (2013) from transporter infected human embryonic kidney (HEK) 293 cells. Values are Ki given as mM (mean ± SD)

Mephedrone has a profound effect on 5-HT, with administration resulting in an increase in extracellular concentration (Kher et al, 2011). 5-HT is a neuromodulator with an important function in many neurobiological processes, including mood; arousal; cognition; pain and temperature regulation (Li and Polter, 2010). There are 14 recognised 5-HT receptors belonging to one of seven families (shown in figure 2).

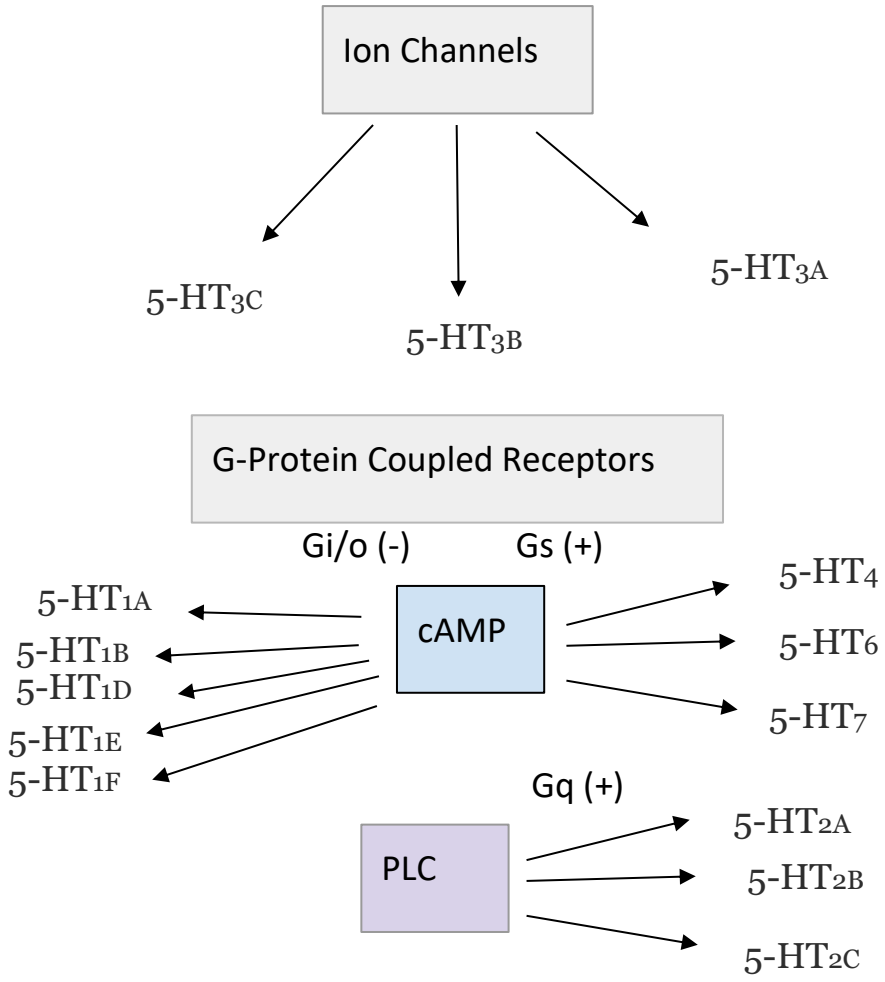

Figure 2. Classification of 5-HT receptor subtypes

The 5-HT family is divided into those which are ion channels (5-HT_{3A-3c}), and the rest are coupled to either a stimulatory, or inhibitory GPCR, which can act to modulate cAMP or PLC. Abbreviations; 3'-5' cyclic adenosine monophosphate (cAMP); phospholipase C (PLC). Gs; Stimulatory G protein, Gi/o, inhibitory G-protein; -, negative; + positive. Information derived from Trang, 2019; Klein and Teitler, 2012; Hoyer, Hannon and Martin (2002).

Serotonin neurons are primarily located in the brain stem, specifically in the dorsal and median raphe with projections to the forebrain and brainstem (Garcia-Garcia, Newman-

Tancredi and Leonardo, 2013). The 5-HT_{1A} receptor is expressed as a pre-synaptic auto receptor, or as a post-synaptic heteroreceptor (Albert and Vahid-Ansari, 2018) and is coupled to an inhibitory G-protein, in which activation of this receptor results in inhibition of 5-HT release via the opening of K^+ channels (Figure 5, Celeda et al, 2004; Garcia-Garcia, Newman-Tancredi and Leonardo, 2013).

5-HT axons have been shown to innervate DA fibres. Many studies have documented the complex relationship between the monoamines, including a study which found lesions of 5-HT and noradrenergic (NE) neurons resulted in increased firing rate of DA neurons in the VTA (Guiard et al, 2008). In the cortex and lateral septum, prolonged cocaine administration increases SERT density whilst simultaneously increasing extracellular 5-HT levels in the cortex (Horne et al, 2008), highlighting the involvement of the serotonergic system in the neurobiological changes that underlie addiction.

1.1.2.1 5-HT_{1A}

The inhibitory role of the 5-HT_{1A} receptor has previously been implicated in the modulation of addiction (Arosen, Bukholt and Schenk, 2016). Postsynaptically, it is found in the highest concentrations in the limbic system (Hannon and Hoyer, 2008), an area which includes the thalamus, hypothalamus, frontal lobe, olfactory bulb, amygdala and hippocampus, and is a known key modulator in many processes (Moseley, 2018). I.V cocaine administration results in a dose-dependent decrease in 5-HT cell firing, (Cunningham and Lakoski, 1988) and induces suppression of 5-HT activity in the dorsal raphe nucleus (Cunningham and Lakoski, 1990), a mechanism thought to be modulated by the 5-HT_{1A} receptor. Supporting this notion,

the 5-HT_{1A} agonist 8-OH-DPAT has been shown to decrease self-stimulation in low doses and increase self-stimulation in high doses, due to the differing pre- and post- synaptic modulation, and WAY-100635 (5-HT_{1A} antagonist) has been shown to block this effect (Ahn et all, 2005). One specific role the 5-HT_{1A} has been shown to influence is the onset of impulsive behaviour, a key symptom of drug addiction, with a reduction in serotonin levels shown to increase impulsivity. This has been documented in both animal (Catalina and Yvon, 2009; Zaichenko, Merzhanova and Vanetsian, 2013) and human studies (Coccaro, Gabriel and Siever, 1990).

The 5-HT_{1A} receptor has also been shown to play a role in the modulation of DA. Agonists of the 5-HT_{1A} receptor increased release of DA, in the ventral tegmental area (VTA) and pre-frontal cortex (PFC), an effect inhibited by WAY-100635 (Diaz-Mataix et al, 2005). The inhibitory control of the serotonergic system by the 5-HT_{1A} receptor has been shown to extend to not only the dopaminergic system, but also the noradrenergic system. Research has found 5-HT_{1A} agonism increases noradrenergic activity in regions implicated in addiction, such as the hypothalamus (Done and Sharp, 1994), hippocampus (Hajos-Korsok and Sharp, 1996) frontal cortex (Thomas, Nutt and Holman, 1998) and VTA (Chen and Reith, 1995), which similarly to the DA system, is inhibited by WAY-100635. Furthermore, 5-HT_{1A} agonists produce a profound increase in activation in the locus coeruleus (LC); the primary source of noradrenergic projections to the forebrain (Hajos-Korsok and Sharp, 1999).

1.1.3 Measures of activation

The immediate early proto-oncogene, c-fos, is a commonly used marker of neuronal activation in rodents (Santos et al, 2017; Shehab et al, 2018). First identified in 1980, c-fos is rapidly expressed (within 5-20 minutes) via coupling to multiple second messenger cascades (shown in Figure 3) (Perrin-Terrin et al,2016). Encoding of this gene leads to the generation of the transcription factor, c-FOS (Kovacs,1998). This transcription factor can trigger the expression of other genes, which in turn mediates long-term changes (Velazquez, Caputto and Boussin, 2015). Studies have used c-fos to identify the brain regions involved in a wide range of stimuli, such as seizures (Dragunow, and Faull, 1989; Krisztin-Peva, Mihlay and Toth, 2019), taste stimulation (Stratford and Thompson, 2016), sleep deprivation (Ledoux et al, 1996) and noxious stimuli (Bullitt, 1990). This also includes drug induced c-fos, which can be induced by multiple neurotransmitter systems (see Figure 3). D_1 receptor activation following i.p. cocaine administration has been shown to induce c-fos expression, as it did not occur in mice lacking functional D_1 receptors (Drago et al, 1996). The use of this marker has identified the striatum, and specifically the Nac and Cp to be important mediators of the effects induced by MDMA (Marie-Claire et al (2003), amphetamine (Lin et al, 1996), methamphetamine (Thiriet, Zwiller Ali, 2001) and cocaine (Lucas, Segu and Hen, 1996). The use of c-fos has also identified the involvement of the pre-frontal cortex in amphetamine administration (Engber et al, 1998). The current study has therefore utilised the expression of c-fos in response to psychostimulants to identify brain regions involved in the mediation of mephedrone, the combination of mephedrone and caffeine and the influence WAY-100635 on this combination treatment.

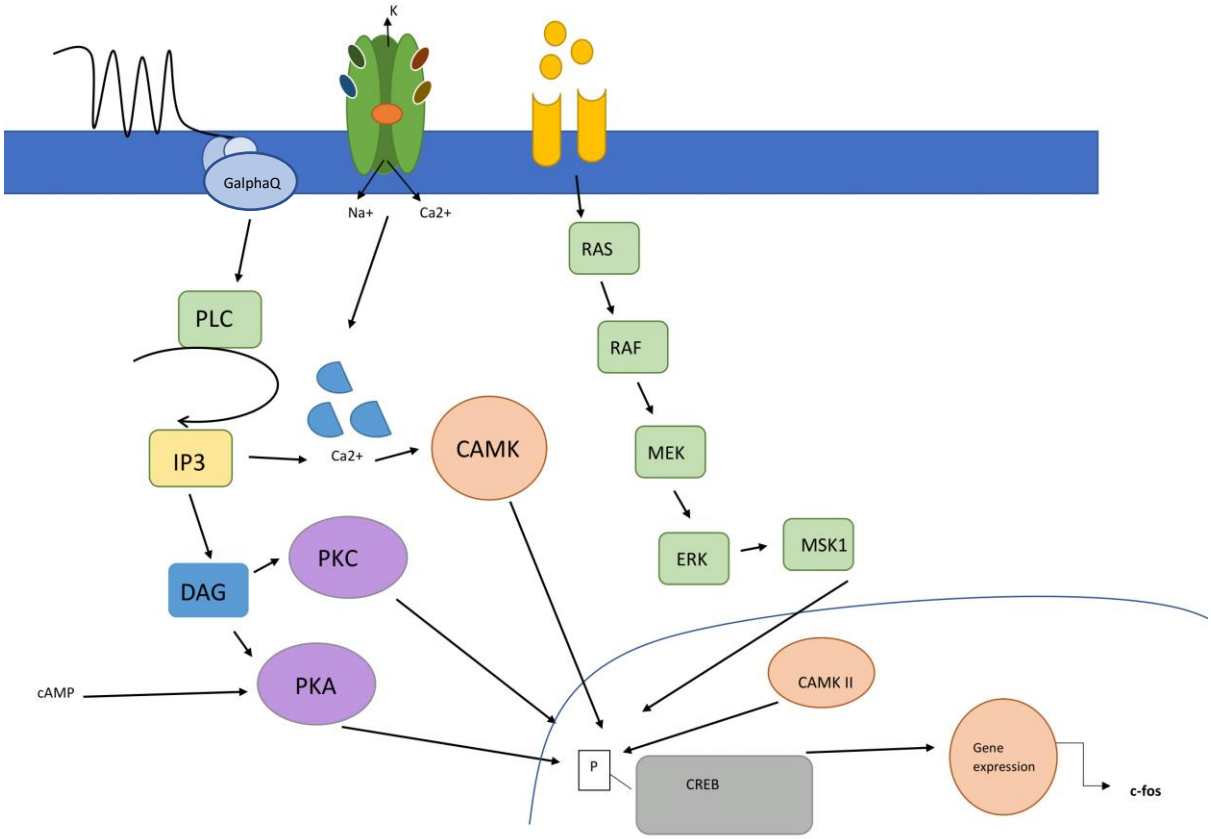

Figure 3. C-fos expression Induced by Multiple Different Second Messenger Cascades

(1). Ligand acts at GPCR resulting in (a) activation of Adenylate Cyclase (AC) which increases the production of cyclic AMP (cAMP). cAMP then enhances Phosphokinase A activity (PKA) which moves to the nucleus and phosphorylates the transcription factor, CREB. (b) GPCR activates Phospholipase C (PLC) which promotes the hydrolysis of Phosphatidylinositol 4,5-bisphosphate (PIP2) into 1,4,5-trisphosphate (IP3) and Diacylglycerol (DAG). DAG can then activate either PKC or PKA, which then phosphorylates CREB. IP3 acts at intracellular calcium stores increase intracellular calcium which results in the activation of cam kinase II, which can move into the nucleus and phosphorylate CREB. (2) Ligand acts at NMDA receptor, which removes the mg^{2+} blockade resulting in an influx of Sodium (Na^{+}) and Calcium (Ca2+). A high intracellular concentration of calcium results in the activation of Cam Kinase II (CAMK), which moves into the nucleus and phosphorylates CREB. (3) Growth factors act at tyrosine kinase

receptors, which activate the RAS cascade. MSK1 results in phosphorylation of CREB. (3) The phosphorylation of CREB results in its activation as a transcription factor in gene expression regulation, resulting in *c-fos* gene expression.

Brain Region	**MDMA**	**Cocaine**	**Amphetamine**
Pre-frontal cortex	✓	✓	✓
Nucleus Accumbens	✓	✓	✓
Striatum	✓	✓	✓
Hippocampus	✓	✓	

Table 4. C-fos expression seen in previous rodent studies

(Marie-Claire et al,2003 ; Johannason et al, 1994 ; Lucus, Sequ and Hen, 1996 ; Stephenson, Hunt, Topple and Mcgregor, 1999 ; Lin et al, 1996; Colussi-Mas et al, 2007)

1.1.4 Preclinical Studies

1.1.4.1 Effect on Locomotor effects and behaviour

It is well known that drugs of abuse enhance locomotor activity (Boys, Marsden and Strang, 2001). Cocaine administration has been shown to induce an increase in locomotor activity (Padovan-Hernandez and Knackstedt, 2018; Koeltzow and Vezina, 2005; Perrine et al, 2015; Fleckenstein, Boka and Kuhar, 1996), and similar findings have been seen with MDMA (Musolino et al, 2019; Varela et al, 2011) and amphetamine (Guaita et al, 2018) administration.

The nigrostriatal pathway is thought to be involved in locomotor activity, and due to it being highly innervated by dopaminergic neurons, the primary target for these drugs, it is therefore expected that drugs of abuse induce such an effect. Previous studies have documented increased locomotor activity in response to mephedrone administration (Sichova et al, 2018;

Motbey et al, 2012; Kohler, Cibeli and Baker, 2019; Martínez-Clemente et al, 2013; Shorthall et al (2015). Mephedrone induces hyperactivity in a similar manner to MDMA and amphetamine (Motbey et al, 2012), and has been found to enhance locomotor activity with both acute dosing (Miller at all, 2013; Wright et al, 2012) and chronic dosing (Shorthall et al, 2015), in a dose dependent manner (Šíchováet al, 2018). In line with the pharmacokinetics mentioned previously, many studies reported that the increased locomotor activity displayed a rapid onset and short duration (Shorthall et al, 2015; Šíchová et al, 2018; Papaseit et al, 2020), especially when compared with other drugs of abuse, including cocaine (Kohler, Cibeli and Baker, 2019)**,** MDMA and methamphetamine. Furthermore, a high ambient temperature has been found to increase locomotor activity as Miller et al (2013) found significantly increased locomotion at 30°C compared with 20°C room temperature. Shorthall et al (2015), found that increased locomotor activity resulting from mephedrone administration is attenuated by both a 5-HT depletion and 5-HT_{1B} antagonism, highlighting the involvement of the serotonergic system in mephedrone induced hyperactivity. As Mephedrone is often taken in nightclubs with a high temperature, it is extremely important to understand the effects of ambient temperature on mephedrone usage.

1.1.4.2 Effect on temperature and cardiovascular function

Stable body temperature is regulated by homeostasis, and it is vital for normal physiological processes to occur. Hyperthermia relates to increased body temperature, whereas decreased body temperature relates to hypothermia. The effects of hyper- and hypothermia can result in fatigue, dizziness and vomiting, but can also induce lethal effects such as brain damage and other vital organ failure. Thermoregulation is controlled via the hypothalamus, specifically within the temperature sensitive preoptic area of anterior hypothalamus (POA) neurons are thought to play a vital role (Wang et al, 2019).

Drugs of abuse can have a significant effect on thermoregulation. For example, previous research has found amphetamine induces a hypothermic effect in rats (Yehuda, Carrosso and Ben-Uriah (1981); Yehunda et al (1980). Similar to effects seen in locomotion, the ambient temperature is also an important factor in drug induced temperature changes (Summarized in table 4).

Drug	**Ambient temperature**	**Effect**
Cocaine	Normal room temperature	Reduction in core body temperature
	High ambient temperature	Hyperthermia
Amphetamine	High ambient temperature (30°C)	Hyperthermia
	Low ambient temperature (7°C)	Reduction in core body temperature
MDMA	lower ambient temperature (20°C-22°C)	Hypothermia
	high ambient temperature (28°C-30°C)	Hyperthermia

Table 4. Effects of psychostimulants on body temperature

(Data obtained from; Malberg and Seiden, 1988; Gonzalez, 1993; Borbely, Baumann and Waser, 1974)

Previous research has identified the ability of mephedrone to mediate thermoregulation (Šíchová et al, 2018, Shorthall et al 2015, Wright et al, 2012), which is in line with effects

reported by recreational users of mephedrone. For example, recreational users reported red/blue skin, cold and numb extremities and body sweating, alongside uncomfortable changes in body temperature regulation (Winstock et al, 2011, ACOID, 2010).
However, preclinical studies identifying the effects of mephedrone on thermoregulation have produced controversial results, with some identifying a hypothermic response (Shorthall et al, 2012; Shorthall et al, 2015; Raúl López-Arnau et al, 2015), and others, a hyperthermic response (Šíchová et al, 2018 ; Zona, Grecco and Sprague, 2016; Baumann et al. 2012). The difference in findings may be attributed to the ambient temperature the studies are carried out in, alongside species and strain differences, and differential dosage patterns.

Previous research has highlighted the involvement of the serotonergic system in thermoregulation (Myers, 1981; Schwartz et al, 1995). For example, Shorthall et al (2015) found that WAY- 100635, a 5-HT_{1A} antagonist attenuated mephedrone induced hypothermia. Similar research has reported supporting findings, for example Yang et al (2017) and Agurrire et al (1998) found antagonism of the 5-HT_{1A} receptor attenuated ethanol, or MDMA induced hypothermia, respectively. Finally, MDMA administered in a cold ambient temperature reduces core body temperature and tail temperature, in which the latter is attenuated by WAY-100635 administration (Rusyniak, Ootsuka and Blessing, 2008). Mechan et al (2001) reported MDMA induced hyperthermia, however the 5-HT_{1A} antagonist 8-OH-DPAT did not influence MDMA induced thermoregulation. The difference in findings may be due to the fact the WAY-100635 acts selectively at the 5-HT_{1A} receptor, whereas the 8-OH-DPAT displays full antagonist properties.

1.1.4.3 Relevant brain areas

Previous research has implicated several brain regions as being important in mediating the effects of recreational drugs such as mephedrone and the other drugs previously mentioned. One region that has shown to be particularly important is the prefrontal cortex, a region known to modulate higher cognitive functions. Specifically, the medial pre-frontal cortex (mPFC) contains a high amount of neurotransmitter systems, as there are a number of serotonergic, noradrenergic and dopaminergic afferents in the mPFC (Steketee, 2002), all which mephedrone has a high affinity for (See section 1.1.3). It is therefore no surprise that previous mephedrone research has further identified the mPFC as an area that mediates mephedrone, with administration (25 mg/kg, s.c) resulting in increased release of DA and 5-HT in the mPFC (López-Arnau et al, 2018).

The striatum is functionally divided into two components, the dorsal and ventral striatum. The dorsal striatum consists of the caudate nucleus and putamen (Cp) and receives dopaminergic input from the substantia pars compacta (Haber, 2014). Methamphetamine has been shown to alter the structure of dendritic neurons in the dorsal striatum (Jedynak et al (2007), suggesting it may be a key site of action for psychostimulants. Similary, the ventral striatum is associated with the limbic system, a region vital for feelings of reward and motivation in response to mephedrone and similar psychostimulants. In addition, the ventral striatum also includes the nucleus accumbens core (NacC) and shell (NacS). Previous research has demonstrated distinctly different roles the NacC and NacS mediates in addiction (Schippers et al, 2017; Spoelder et al, 2016), which can be attributed to the differing innervations of each subregion and their respective roles. For example, the NacC receives projections from the basal ganglia, a region dense in projections from multiple other

subregions. It projects to the dorsolateral ventral pallidum, which has been shown to encode motivational information and conditioned responses (Liu et al, 2020). The anatomical similarities within the NacC and the dorsal striatum have been previously reported, as these two regions share multiple characteristics (Castro and Bruchas, 2019). The NacS receives direct innovations stemming from the lateral hypothalamus and other regions (shown in figure 29). The specific roles of these two regions have been examined by selective inhibition or lesion. For example, Ambroggi et al (2011) reported that NacC inhibition (via blockade of excitatory transmission by AMPA and NMDA antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2-amino-5-phosphonopentanoic acid) resulted in an impairment in tasks which are mediated by sensory cues, implicating the role the NacC plays in responding to motivational information. However, inhibiting the NacS results in an increased response to unrewarding stimuli, highlighting the role the NacS plays in the reinforcement of rewarding stimuli, which along with the results from the current study, suggests mephedrone is highly rewarding. The role the Nac plays in addiction is well documented (Zhu et al, 2016; Charrette et al, 2002), and psychostimulants have been previously shown to induce DA release within the Nac, (Chiara et al, 1999; Saddoris; 2016; Yorgason et al, 2020; Kalivas and Duffy, 1990; Pettit and Justice Jr, 1989), which is well known to induce the reinforcing effects caused by administering addictive drugs. In response to mephedrone, MDMA and amphetamine, the extracellular levels of DA and 5-HT in the NacS were increased compared with basal levels, highlighting the importance of this region (Kehr et al, 2011).

The final region of interest that will be vital to this study is the hypothalamus. Psychostimulants such as mephedrone alter body temperature and can cause hypo- or hyper-thermia. The hypothalamus controls body temperature, with around 30% of its neurons being sensitive to temperature change (Wang et al, 2019) and therefore psychostimulants may act

upon this area, producing the thermoregulatory effects seen in rat studies and recreational users.

1.1.5 Clinical studies

1.1.5.1 Pharmacodynamics

Few studies have researched the pharmacodynamics of mephedrone in human users, however, a small amount of data is available. A study conducted on 12 healthy male subjects (aged 21-39 years) were orally administered mephedrone (200mg), MDMA (100mg) or placebo, before psychomotor, physiological, pharmacokinetic, and subjective data was collected. The findings showed an increase in blood pressure (both systolic and diastolic), heart rate, and psychomotor responses. In terms of the subjective data, participants reported euphoria and changes in perspective (Papaseit et al, 2016). As previously mentioned, mephedrone has a wide range of effects considered to be desirable in recreational users, such as euphoria, well-being, feelings of pleasure alongside mild changes in perception (Papaseit et al, 2020), which have been described to be similar to those experienced with MDMA, amphetamine and cocaine (Rácz et al, 2012; Zawilska et al, 2013). However, the adverse effects of both acute and long-term mephedrone on behaviours such as anxiety, anger, paranoia and craving are less clear (Winstock et al, 2011; Jones, Reed and Parrott). It is important to recognise that these self-reported effects come from the illicit use of impure drugs from the UK party scene, so the findings may not accurately reproduce the symptoms of mephedrone nor be generalizable to other forms of use. None the less, similar findings have been reported in studies using rats, with acute administration resulting in increased levels of anxiety (Budzynska et al, 2015) and longer term mephedrone has been reported in some studies to cause a depletion in 5-HT (Motbey et al, 2012), which has been implicated in

anxiety and depression (Akimova, Lazenberger and Kasper, 2009). Unlike rats, mephedrone induced 5-HT depletion alongside anxiety-type behaviours are not seen in mice (Hollander et al, 2012) so may be species specific. To increase the likelihood of these results being generalized to humans, the current study used Lister hooded rats.

1.1.5.2 Metabolism

In humans, the CYP2D6 cytochrome is responsible for phase I metabolism of mephedrone, alongside a minor role from NADPH-dependent enzymes (Pedersen et al, 2013) and can be impacted by allelic variation. For example, Olesti et al (2019), found reduced or abolished functionality of the CYP2D6 cytochrome results in higher toxicity, which may account for variation in responses seen in individuals taking mephedrone. It has been recently demonstrated that mephedrone produces metabolites in humans. However, the precence of these in rodents remains unclear. In a clinical trial, 4'-Carboxy-mephedrone and nor-mephedrone were found to be the most abundant mephedrone metabolites found in plasma (Olesti et al, 2019). Mayer et al (2016) found the metabolites dihydro mephedrone and nor-mephedrone displayed activity at the DAT, SERT and NET, unlike 4-OH-mephedrone, which was suggested to not cross the blood-brain barrier. Other mephedrone metabolites that have been identified include hydroxyl-mephedrone, alongside 4-carboxy-dihyro-mephedrone and 4-carboy-mephedrone (Pederson et al, 2013). Furthermore, Olesti et al (2019) found correlations in mephedrone plasma concentrations, serotonin blood concentrations and cardiovascular and subjective effects in human subjects.

1.1.5.3 Interactions with caffeine

It is widely documented that drug-drug interactions can have harmful effects in both animals (Kumar et al, 2013) and humans (Leeuwen et al,2013; Ozdemier et al, 1998). Due to drugs of abuse being taken at raves and clubs, they are often co-administered with legal drugs including caffeine and ethanol, due to ease of access of the latter. The administration of caffeine co-administered with psychostimulants has been shown to increase adverse effects in rodents. For example, caffeine has been shown to potentiate the toxicity of cocaine and amphetamine (Derlet, Tseng and Albertson, 1991), alongside MDMA, which has been previously shown to result in exacerbated hyperthermia and 5-HT neuron loss, in which the latter is not seen with MDMA administration alone (McNarmra et al, 2006). Due to the structural similarities of mephedrone and caffeine, it is reasonable to hypothesise that they may interact with each other at the level of recpeptors or signalling pathways.

1.2 Caffeine

Caffeine (1,3,7-Trimethylpurine-2,6-dione) is a naturally occurring methylxanthine, and is the most commonly used psychostimulant world-wide. Primarily sourced from the coffee bean, caffeine is predominantly consumed in beverages, such as coffee and energy drinks (Whickam and Spriet, 2018), inducing its effects (summarised in table 5) primarily via the non-selective antagonism of the A_1 and A_2 receptors (Fredholm et al, 1999).

Subjective effects	Physiological effects
Increased sense of wellbeing	Increased heart rate
Increased energy	Increased respiratory rate
Increased alertness	Increased systolic blood pressure
Increased motivation	Increased diastolic blood pressure
Decreased sleepiness	

Table 5. Effects of caffeine consumption in humans
Data obtained from Childs and de Wit (2006)

1.2.1 Pharmacokinetics

Predominantly consumed from caffeinated drinks, caffeine reaches a peak blood concentration within 30-60 minutes of consumption, and rapidly crosses the BBB, accounting for the fast onset of the stimulant effects felt by consumers after a caffeinated beverage. It is metabolised by the P450 enzyme in the liver. Caffeine is broken down by demethylation to dimethylxanthines, the primary metabolites being paraxanthine, theobromine and theophylline. Due to the long half-life of caffeine, it can stay within the system for up to 10 hours, before being execrated by the kidneys

Parameter	Coffee	Energy drink
C*max* (ug/mL)	3.74 ± 1.09	3.14 ± 0.95
Tmax (min)	59 ± 27	82 ± 42
T1/2 (min)	408 ± 178	399 ± 177
Clearance (mL/min/kg)	1.2 ± 0.6	1.5 ± 0.8

Table 6. Pharmacokinetics of various forms of 160mg caffeine in humans (20 min)
Data obtained from White et al (2016)

1.2.2 Neurochemistry

Caffeine acts primarily on the adenosine receptors. The adenosine receptor family contains 4 receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) (Barkin et al, 2020), all which belong to the GPCR receptor family (Ralevic and Burnstock, 1998). The A_1 receptor is coupled to an inhibitory G-protein ($G_{i/o}$), mediating inhibition of cyclic AMP, with the A_{2A} receptor being coupled to stimulatory G-protein (G_s), thus having opposing effects (i.e. stimulation of cAMP accumulation) (Van Calker, Muller and Hamprecht, 1979). Adenosine is synthesised via several pathways, either via the metabolism of intracellular ATP, ADP and AMP, catalysed by cytoplasmic 5-'nucleotidases, via the action of S-adenosylhomocysteine, and finally, via the extracellular generation of ADP from ATP (Ernst, Garrison and Thompson, 2010). Research has highlighted a range of functions which are mediated by the adenosine receptor family alongside clinical uses for antagonists and agonists of these receptors. Physiological responses include the control of sleep, as previous research has documented adenosine levels increase during prolonged periods of wakefulness, which decrease during sleep (Porkka-Heiskanen, Strecker and McCarley, 2000). Administration of adenosine results in increased sleep, further implicating adenosine in this control. Selective antagonists for the adenosine receptor family are currently in preclinical research to treat disorders such as asthma and Parkinson's disease, and agonists for pathologies including coronary artery disease, different types of leukaemia alongside heart failure and ischemia (Leaker et al,2008; Borea et al, 2018).

1.2.3 Mechanisms of caffeine and mephedrone interaction

Additionally, adenosine receptors are thought to modulate the effects of psychostimulants via the formation of heterodimers with other receptors, which modulate the responses seen with activation of each receptor (Borroto-Escuela et al, 2018). It is well documented that adenosine signalling affects dopaminergic transmission (Meng et al, 2019; Okada, Mizuno and Kaneko, 1996; Quarta et al, 2004), specifically, A_1 receptors are negative modulators of D_1 receptors (Yabuuchi et al, 2006), and so antagonism of these receptors by caffeine may act to increase D_1 receptor signalling, including an increase in cAMP, which would then lead to increased cAMP-dependent responses. This may in part be due to the dimerization between the both the A_{2A} - and the D_2-, and the A1R- and the D1R receptors (shown in figure 4), resulting in allosteric modulation (Guidolin et al, 2019; Borroto-Escuela et al, 2018) and an increase in c-Fos expression. Previous studies clearly support this notion, as A_1 and A_2 receptor agonists decrease striatal dopamine concentration (Okada et al, 1996) and activation of the A_{2A} receptor has been shown to change the affinity of the D_2 receptor to dopamine, as A_{2A} agonists result in reduced DA binding (Ferre et al, 1991). Alongside the DA receptor, the A_{2A} receptor has been found to form dimers with the 5-HT_{1A} receptor (Lukasiewicz et al, 2007), further implicating the adenosine receptor family in the modulation of drug induced monoamine release and consequently, their downstream effects.

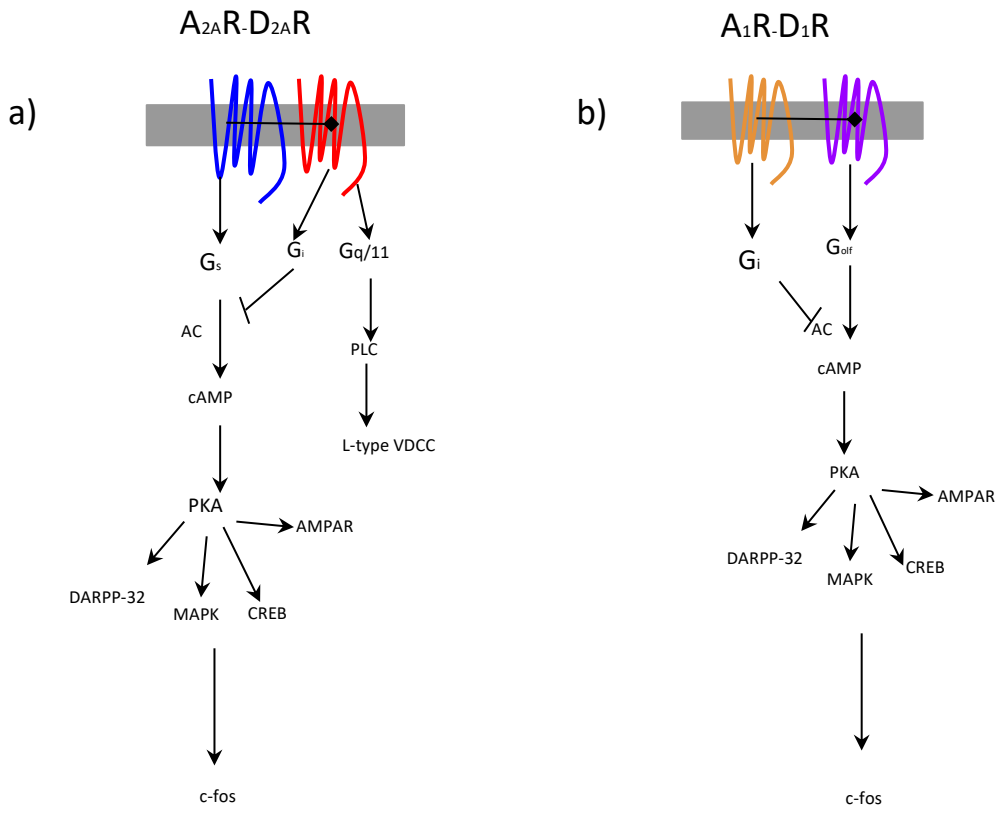

Fig 4. Schematic diagram of the downstream signalling pathways of the $A_{2A}R$-$D_{2A}R$ and A_1R-D_1R heterodimer

A) The $A_{2A}R$ is coupled to a stimulatory G protein (Gs) in which activation results in the activation (indicated by →) of adenylate cyclase (AC), in which stimulates production of protein kinase A (PKA), which then stimulations production of DARPP-32, MAPK and CREB, which ultimately leads to expression of c-fos. The $D_{2A}R$ receptor is coupled to an inhibitory G protein (Gi), in which activation results in inhibition of adenylyl cyclase (indicated by ⊥). When forming a functional heteromer, there is a modulation of the downstream signalling pathway, in which the $A_{2A}R$ decreases binding affinity of dopamine to the $D_{2A}R$

B) The A_1R is coupled to a G_i protein, which acts to inhibit AC. The D_1R is coupled to a stimulatory G_{olf} protein, in which activation stimulates the production of cAMP, which then stimulations production of DARPP-32, MAPK and CREB, which ultimately leads to

expression of c-fos. In a functional heteromeric, the A_1R counteracts the D_1 mediated activation. (Information derived from Ferré et al, 2007)

1.2.4 Relevant brain areas

Previous studies using c-fos have identified regions activated by caffeine. A dose of 75 mg/kg induced expression across the striatum and nucleus accumbens, hypothalamic nuclei and the paraventricular hypothalamus (Bennett and Semba, 1999). Research has also found caffeine administration increases DA in the PFC (Acquas et al, 2002), implicating this region in mediating the effects of caffeine. The same study found no increase in DA in the NacC or NacS, which contrasts to other psychostimulants, which produce dramatic increases in monoamines in these brain regions.

1.3 Experimental approach and justifications

Previous studies have demonstrated that mephedrone influences locomotor hyperactivity and hypothermia, which are attenuated and briefly prevented by pre-administration of the 5-HT_{1A} antagonist WAY-100635. Furthermore, when mephedrone is combined with caffeine, the coadministration of these drugs enhances mephedrone induce hyperactivity and converts hypothermia to hyperthermia, whilst also inducing stereotyped behaviours that suggest serotonin syndrome, both which have an increased risk on health. As many clubbers ingest highly caffinated drinks alongside recreational drugs, alongside the known adverse interaction of caffeine with stimulant drugs of abuse, the effect of coadministration of caffeine and mephedrone, alongside the influence of 5-HT_{1A} antagonism is examined in this thesis. C-fos is used to examine the effects of the drugs as previous research has found it a useful tool in the identification of cells that respond to psychostimulants.

***In vivo* work was performed by a previous PhD student within the laboratory group.** In brief, 48 male Lister Hooded rats (Charles River, UK) implanted with striatal microdialysis probes (CMA Microdialysis AB) and subcutaneous temperature sensing microchips (iDENTICHIP; Animalcare) received i.p. vehicle (saline 1mL/kg) or WAY-100635 (0.5mg/kg) then 30min later vehicle, caffeine (10mg/kg), mephedrone (10mg/kg) or caffeine plus mephedrone (10mg/kg each). Temperature, locomotor activity and stereotyped behaviour were monitored for 120 min alongside dialysate collection. This work reported short-term hyperlocomotion (5-15min) and hyperthermia (15-30min), was observed after mephedrone or caffeine (baseline +0.29±0.13/+0.53±0.20°C; $P<0.05$ versus vehicle). Co-administration of caffeine and mephedrone prolonged locomotor activity (55-65min) and hyperthermia (by 30 min) (baseline +0.65±0.17°C; $P<0.05$-0.01 versus vehicle/mephedrone alone). Stereotyped behaviour (head weaving/forepaw treading) was induced by mephedrone; however, caffeine had no effect. WAY-100635 had no effect on any of the behavioural and physiological responses to mephedrone and caffeine co-administration.

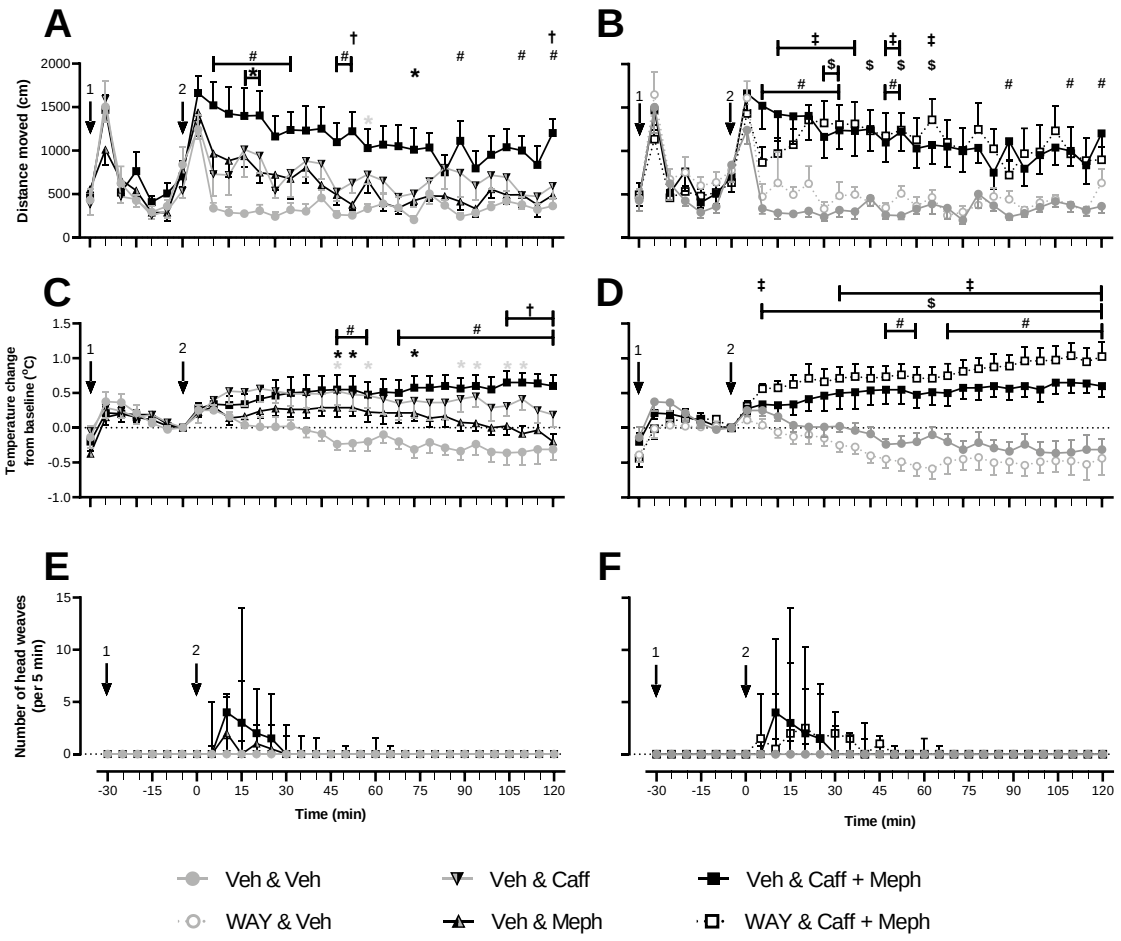

Figure 5. Effect of A, C, E caffeine co-administration on the time course of mephedrone-induced behavioural and physiological changes, and B, D, F of the 5-HT1A receptor antagonist WAY-100635 on the time course of the combined syndrome. Mean ± SEM A-B distance moved and C-D change in subcutaneous temperature, together with median ± interquartile range E-F counts of lateral head weaving during striatal microdialysis. Singly housed adult male Lister hooded rats received i.p. pre-treatment (arrow 1) with saline vehicle (1 mL kg-1; Veh) or WAY-100635 (0.5 mg kg-1; WAY) at -30 min, followed (arrow 2) by i.p. treatment with saline vehicle (1 mL kg-1; Veh), caffeine (10 mg kg-1; Caff), mephedrone (10 mg kg-1; Meph), or caffeine plus mephedrone (10 mg kg-1 each in the same injection; Caff + Meph) at 0 min (n=8 per pre-treatment x treatment combination). *P<0.05 Veh & Caff, *P<0.05 Veh & Meph, and #P<0.05 Veh & Caff + Meph versus Veh & Veh; †P<0.05 Veh & Caff + Meph versus Veh & Meph; ‡P<0.05 WAY & Caff + Meph versus

Veh & Veh; $P<0.05 WAY & Caff + Meph versus WAY & Veh. Locomotor and temperature data were analysed by three-way repeated-measures ANOVA with Tukey's multiple comparison post-hoc. Lateral head weaving had a median and variance of zero in the absence of mephedrone and these data were not included in statistical analyses. Remaining combinations were compared using planned pairwise Mann-Whitney U tests. ***This Figure and legend were provided in their entirety by Luke O'Hara, who acquired and analysed these data as part of his PhD, prior to the start of this MRes.***

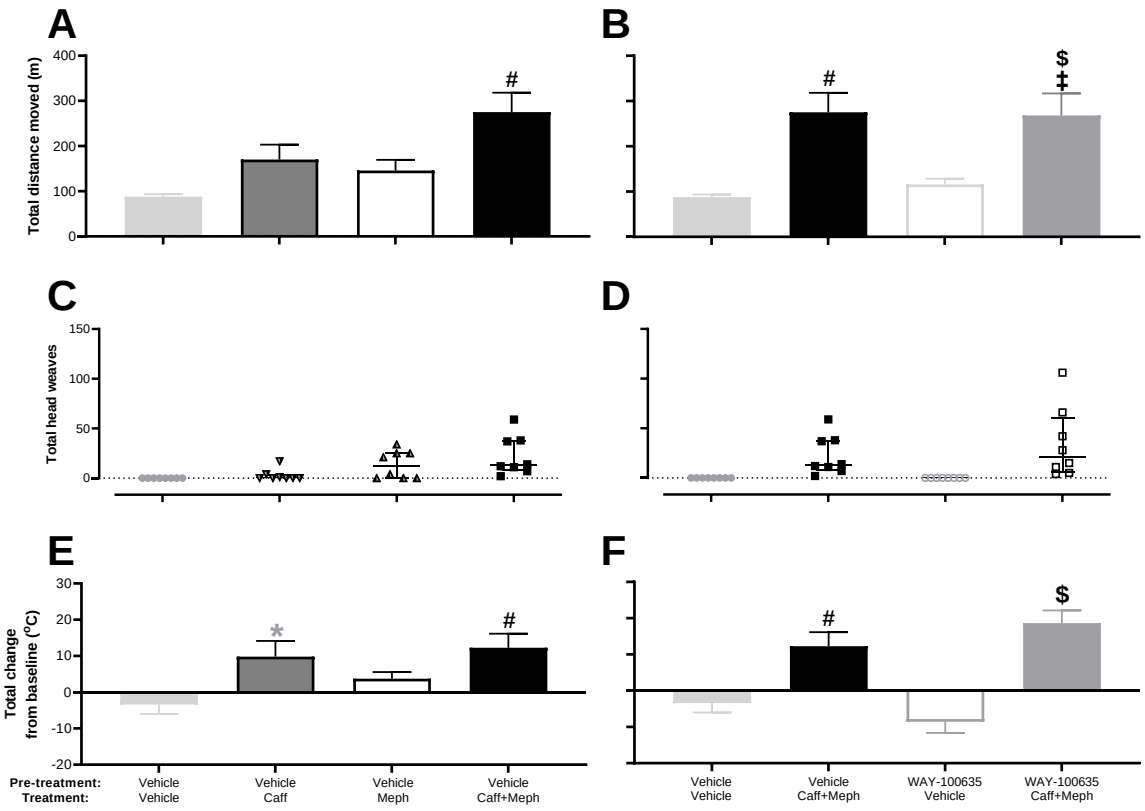

Figure 6. Effect of A, C, E caffeine co-administration on total mephedrone-induced behavioural and physiological changes, and B, D, F of the 5-HT1A receptor antagonist WAY-100635 on the total combined syndrome. Mean ± SEM A-B total distance moved and C-D cumulative change in subcutaneous temperature, together with median ±

interquartile range E-F counts of lateral head weaving during striatal microdialysis. Singly housed adult male Lister hooded rats received i.p. pre-treatment with saline vehicle (1 mL kg-1; Veh) or WAY-100635 (0.5 mg kg-1; WAY), followed 30 min later by i.p. treatment with saline vehicle (1 mL kg-1; Veh), caffeine (10 mg kg-1; Caff), mephedrone (10 mg kg-1; Meph), or caffeine plus mephedrone (10 mg kg-1 each in the same injection; Caff + Meph). Totals are for the 2 h period following this second injection (n=8 per pre-treatment x treatment combination). *P<0.05 Veh & Caff, *P<0.05 Veh & Meph, and #P<0.05 Veh & Caff + Meph versus Veh & Veh; †P<0.05 Veh & Caff + Meph versus Veh & Meph; ‡P<0.05 WAY & Caff + Meph versus Veh & Veh; $P<0.05 WAY & Caff + Meph versus WAY & Veh. Locomotor and temperature data were analysed by two-way ANOVA with Tukey's multiple comparison post-hoc. Lateral head weaving had a median and variance of zero in the absence of mephedrone and these data were not included in statistical analyses. Remaining combinations were compared using planned pairwise Mann-Whitney U tests.

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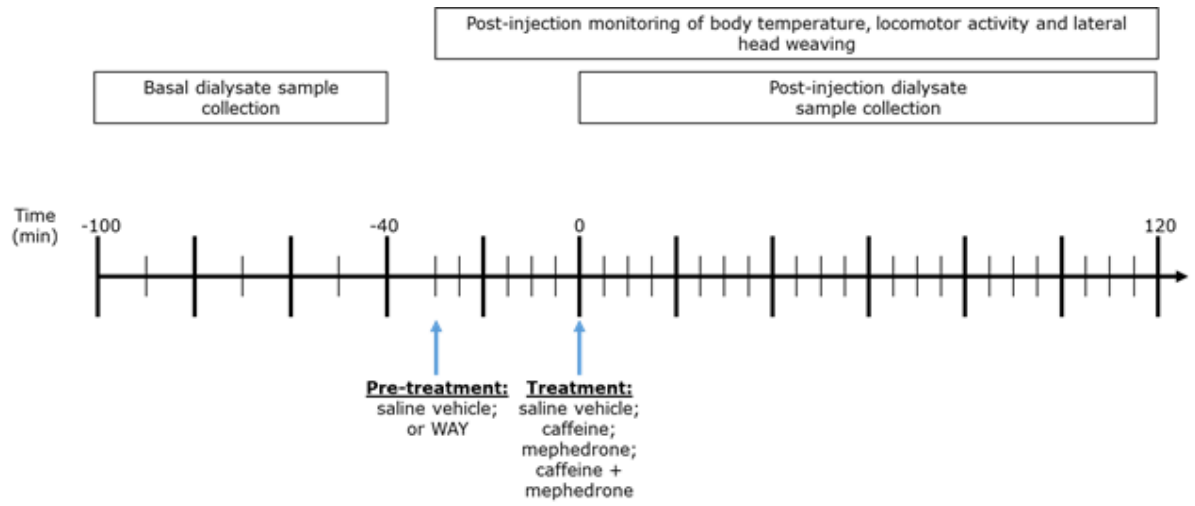


Figure 7. Design of the current study

The doses and experimental design were used in accordance with previous studies researching the neuropharmacology of mephedrone. For example, the mephedrone dose (10mg kg^{-1}) was chosen as it has been previously shown to induce locomotor activity (Green et al, 2014), and the caffeine dose has been previously shown to enhance mephedrone induced locomotion, whilst converting the mephedrone induced hypothermia to hyperthermia (Shortall et al, 2016). The experimental design also involved a double blinding of the data, alongside a total of 6 treatment combinations **(**in Table 5) to analyse the interaction between caffeine and mephedrone, alongside the effect of WAY-100635 on this interaction, to examine whether activation of the 5-HT_{1A} receptor contributes to the enhanced mephedrone-induced behaviour and thermoregulatory response.WAY-100635+ caffeine, and WAY-100635+Mephedrone were not included as treatment groups, as the current study aims to investigate only the interaction of caffeine and mephedrone, and the influence of WAY-10-635 on the combined effect of caffeine and mephedrone. Although this resulted in an unbalanced design, it allowed a reduction of 16 animals used overall in the experiment. Due to this unbalanced design, the data were analysed and presented as two subgroups to identify firstly if there is an interaction between combined treatment of caffeine and mephedrone, and secondly to identify if this combined effect is influenced by WAY-100635. A pilot study was conducted to determine the optimal antibody concentration to observe c-fos expression, whereby the protocol was conducted with varying concentrations of antibody (from 1:2500 – 1;10,000). In addition, c-fos was used to measure the density of cells responding to caffeine and mephedrone, as this is an established marker to identify neuronal activation following psychostimulant administration.

As mentioned previously, rats have a closer neurochemical response to drugs of abuse than of mice, and due to this, rats were chosen in this study. Alongside this, due to the use of

microdialysis probes, the larger brain size seen in rats is more suited to the project as the placement of the probe and specific brain regions will be easier to identify. However, there are limitations in choosing rats over mice, mainly due to their larger size which results in increased space and food, thus increasing overall costs. The larger size of rats also results in a higher dosage needed, which again, increases the overall costs of the study.

Treatment combinations used	Treatment combinations **not used** to reduce animals used in experiment
Vehicle + Vehicle	WAY-100635 + Caffeine
Vehicle + Caffeine	WAY-100635 + Mephedrone
Vehicle + Mephedrone	
Vehicle + Caffeine & Mephedrone	
WAY-100635 + Vehicle	
WAY-100635 + Caffeine + Mephedrone	

Table 6. Possible treatment combinations of the current study

The prefrontal cortex, striatum and hypothalamus were chosen for staining, due to their known involvement with drugs of abuse which is stated in past literature (Marie-Claire et al,2003; Johannasoon et al, 1994; Lucus, Sequ and Hen, 1996; Stephenson, Hunt, Topple and McGregor, 1999; Lin et al, 1996; Colussi-Mas et al, 2007). The doses used are in alignment with previous studies (Shorthall et al, 2016).

1.4 Aims

There is an established issue within the preclinical and clinical literature surrounding the co-consumption of caffeine and mephedrone. Therefore, the current study aims to identify the specific brain regions involved in mephedrone and caffeine induced hyperthermia, and the pathways involved in the reward response associated with mephedrone

administration. Identifying the neuronal pathways responsible for the control of thermoregulation and reward after co-administration of mephedrone and caffeine will be an important advancement for neuropharmacological research and to ensure harm reduction in those taking mephedrone recreationally. Alongside this, identifying the brain regions involved in reward will allow researchers to develop a complex understanding of the mechanisms behind adverse effects resulting from mephedrone administration and provide specific targets for novel drugs to alleviate the devastating symptoms caused by intake and addiction. Finally, identifying the neuronal pathways involved in the combination treatment of mephedrone, and caffeine induced hyperthermia that was studied in the previous research described will be essential for the development of treatments for drug induced hyperthermia.

1.5 Hypotheses

The primary hypotheses in this thesis are:

I. Mephedrone will induce c-fos expression in all the brain regions previously identified as sensitive to psychostimulants

II. Caffeine will enhance the mephedrone induced c-fos expression

III. Inhibition of the 5-HT_{1A} receptor by WAY-100635 will attenuate the effects induced by this combination of mephedrone and caffeine

2.0 Experimental procedures

2.1 Sectioning

C-fos immunochemistry was performed as described in Jones et al (2014), as this has proved to be a well validated method for measuring c-fos expression.

The cerebellum of each brain was carefully sliced off in the coronal plane using a blade with the ventral surface aligned to the horizontal plane, then the cortex placed on a microtome stage encased in optimal cutting temperature (OCT), and cryofreeze to facilitate freezing. The brain was then coronally cut into at 60μm sections, with each slice being placed in phosphate buffered saline (PBS) to view before being stored in an 80 well tray, with each well containing a total of 6 slices and 0.75ml antifreeze mix (200ml PBS, 150ml ethylene glycol and 150ml glycerol), before being placed in the freezer at -20°C.

2.2 Immunohistochemistry

Sections (6 per animal, per well) were washed in PBS (3 x 5 minutes), before being placed in 3% H_20_2 in PBS to block endogenous peroxidase and prevent interference with the DAB protocol. The sections were then washed again in PBS (2x5 minutes) before placed in 2% blocking buffer containing normal goat serum and day 1 buffer (30mg 0.3% BSA, 10μl 0.1% Triton-X in 10ml 0.1 M PBS) for one hour at room temperature to reduce non-specific background staining. Finally, sections were incubated overnight at 4°C in primary Antibody (rabbit anti-c-fos; Abcam; dilution of 1:2500 in day 1 buffer).

Day 2 buffer was prepared (0.3% BSA, 0.1% tritonX in 0.1M PBS) and sections were washed in it (3 x 5 minutes) before placed in the biotinylated secondary antibody (vectastain

ABC Elite Kit; Vector Labs, Burlingame, CA, USA) for one hour. After 45 minutes the ABC solution was made using the ABC kit (vectastain ABC Elite Kit; Vector Labs, Burlingame, CA, USA) and left to incubate for half an hour. After the incubation period, the sections were then washed in day 2 buffer (3 x 5 minutes) before being placed in the previously prepared ABC solution. After incubation (1 hour), sections were sequentially washed in day 2 buffer (1 x 5 minutes) and PBS (2 x 5 minutes). Immunodetection was performed using diaminobenzene (DAB), in which the solution was created according to the kit instructions (DAB substrate Kit, Peroxidase (HRP) with Nickel (3,3'-diaminobenzidine, Vector Labs) and sections incubated in it (5 min). The sections were then mounted onto slides using dibutyl phthalate polystyrene xylene (DPX), before being placed in 75% ethanol, 95% ethanol, 100% IMS and finally xylene, each for 10 minutes. After being allowed to dry in the fume hood, the antigen-antibody complex was visualized under a microscope. To allow for a control to ensure the specificity of the staining, some sections were incubated without primary antibody.

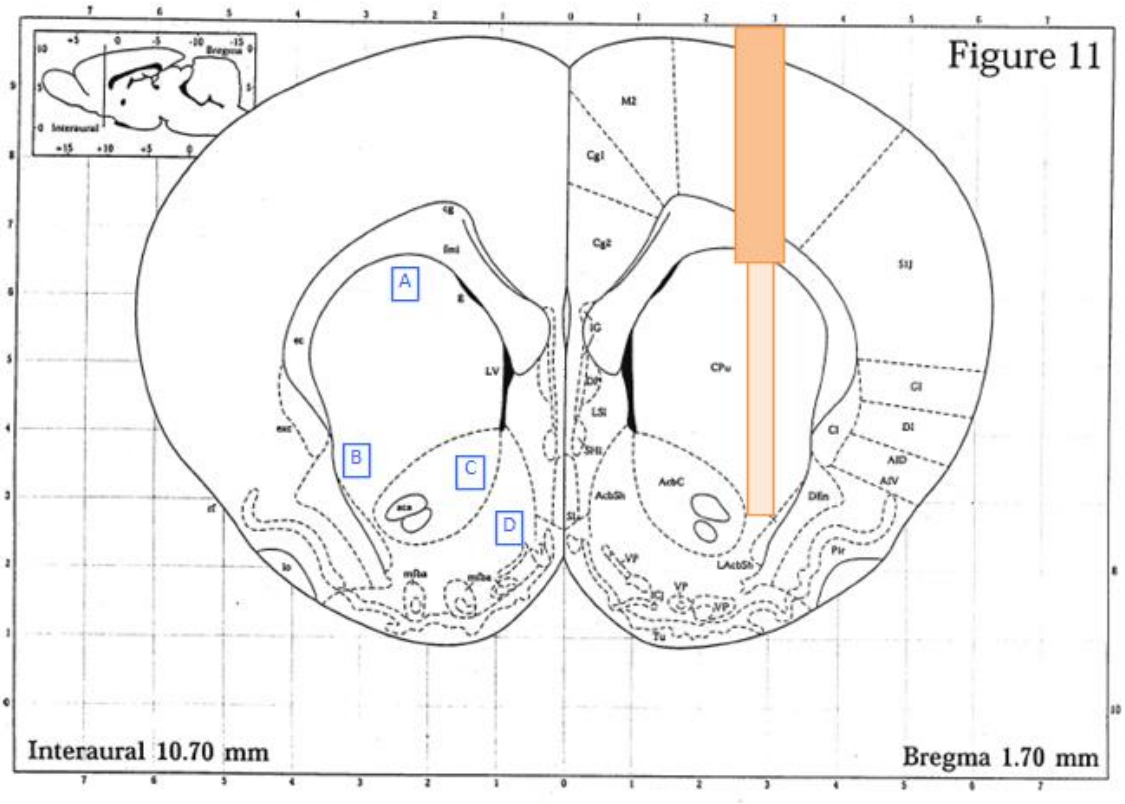


Figure 8. Stereotaxic atlas of the rat brain (Paxinos & Watson, 1998) in the coronal plane at the level of the striatum.

A microdialysis guide cannula and probe were positioned in the right hemisphere (indicated dark and pale orange shading, respectively). c-Fos immunohistochemistry used tissue from the left hemisphere and images were obtained from consistently placed regions of interest (indicated by blue rectangles) in the **A** dorsal striatum, **B** ventral striatum, **C** nucleus accumbens core and **D** nucleus accumbens shell.

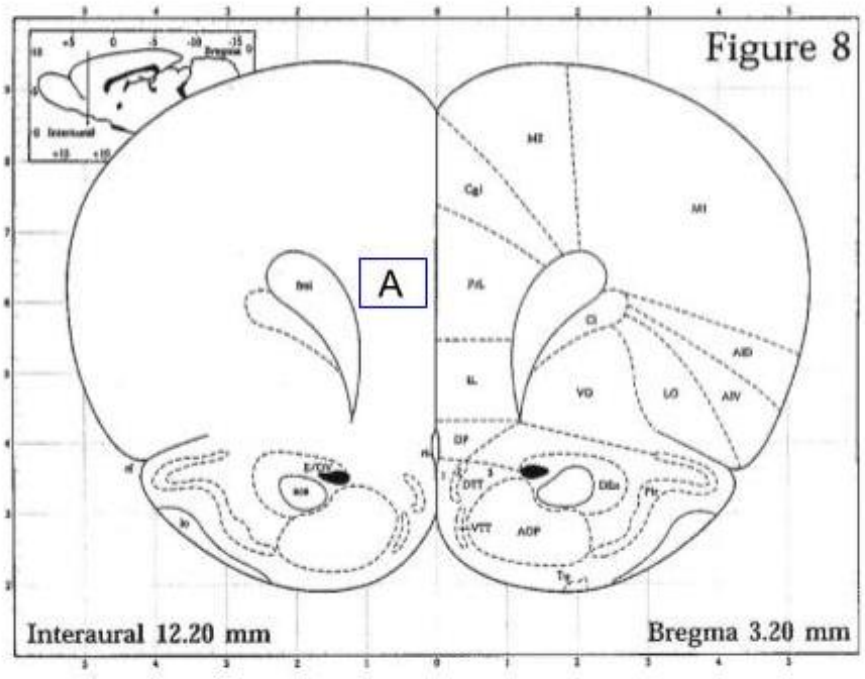


Figure 9. Stereotaxic atlas of the rat brain (Paxinos & Watson, 1998) in the coronal plane at the level of the prefrontal cortex

c-Fos immunohistochemistry used tissue from the left hemisphere and images were obtained from consistently placed regions of interest (indicated by blue rectangles) in the **A** Prelimbic cortex

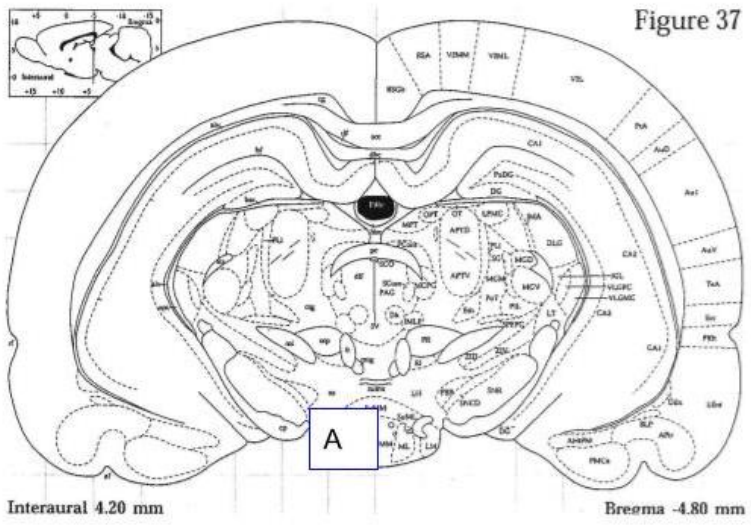
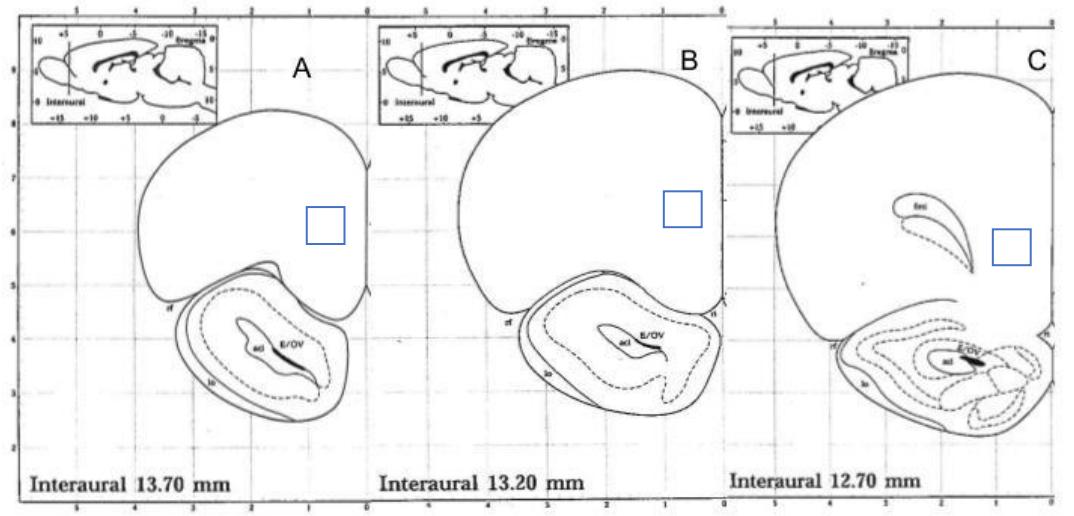
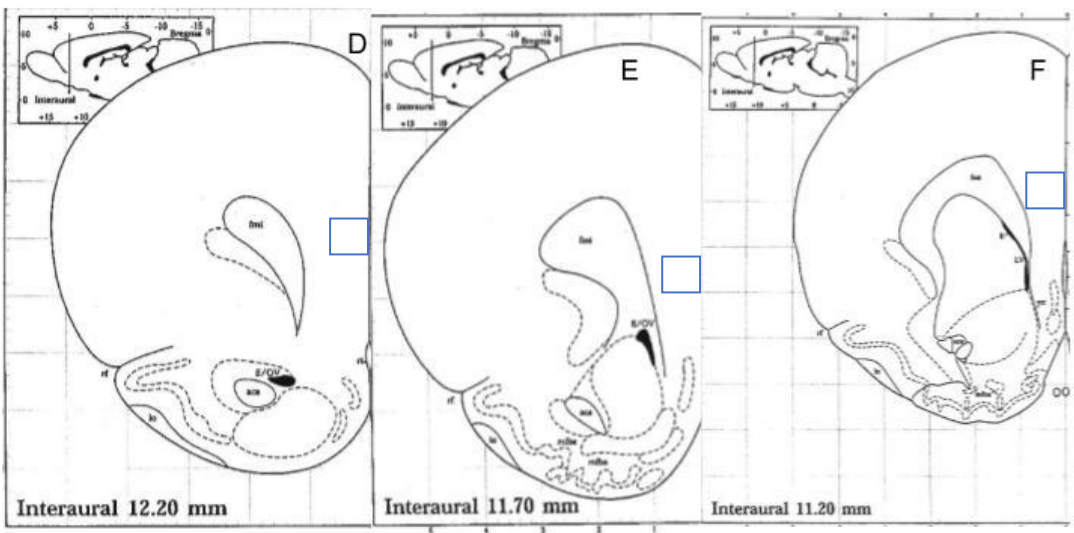


Figure 10. Stereotaxic atlas of the rat brain (Paxinos & Watson, 1998) in the coronal plane at the level of the hypothalamus

c-Fos immunohistochemistry used tissue from the left hemisphere and images were obtained from consistently placed regions of interest (indicated by blue rectangles) in the **A** hypothalamus





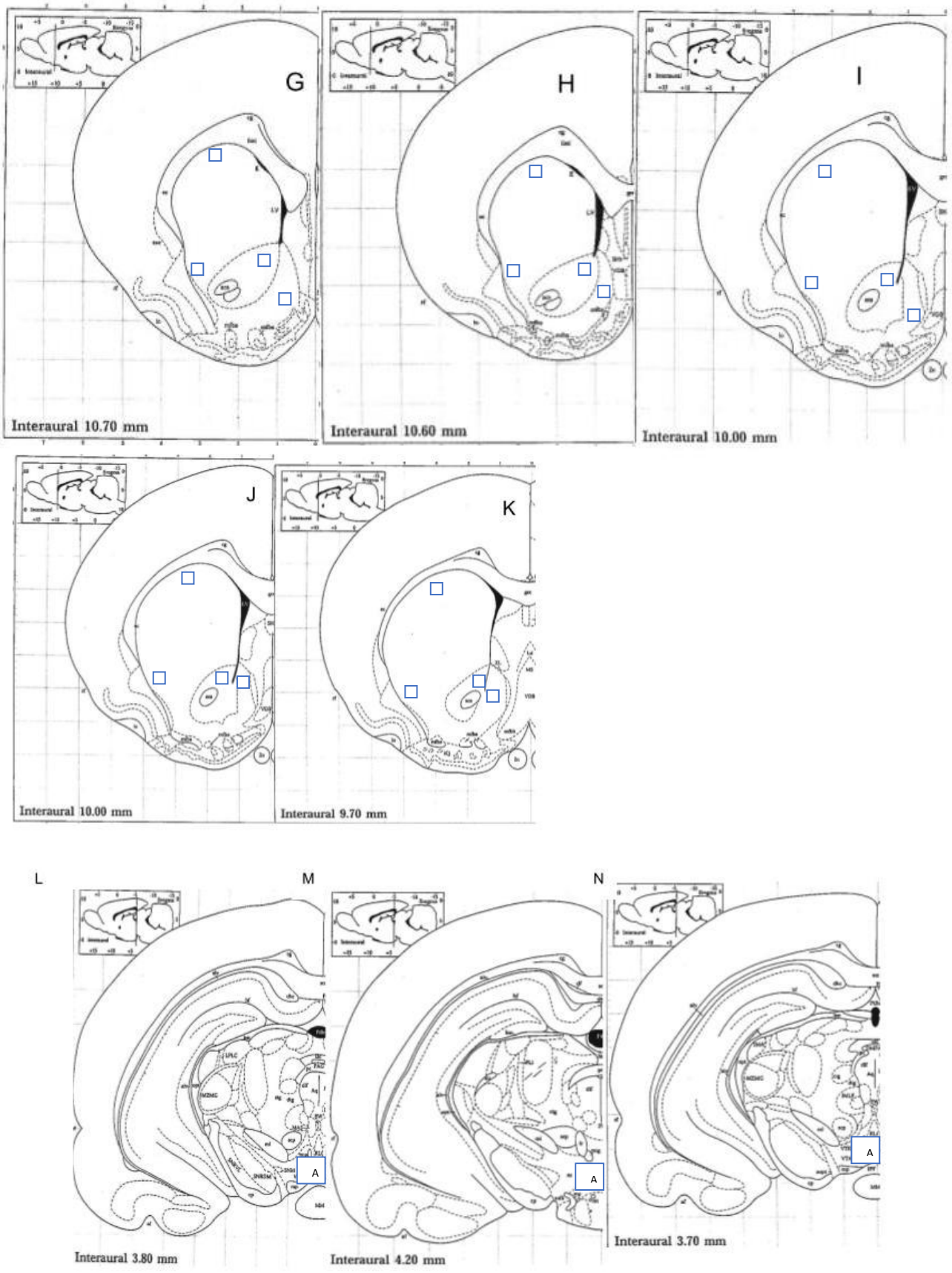


Figure 11. Diagram of rat brain areas chosen for c-fos immunohistochemistry

Coronal sections measured from Bregma as indicated from Paxinos and Watson (1986) for the measurement of c-fos expression. A-D represents sections used for the pre-frontal cortex, E-K for the striatum, and L-N for the hypothalamus. Blue squares indicate the areas of interest.

2.3 Microscopy and automated cell counting

The regions of interested were confirmed using Paxinos and Watson's stereotaxic atlas (Paxinos and Watson, 1986), which include the pre-limbic cortex n the PFC (figure 4A-D), the striatum (Figure 4e-k), and the hypothalamus (L-N). These specific regions were chosen due to previous literature (Schatz et al, 2000; Martin-Garcia et al, 2014; Hunt et al, 2011) and known effects of drugs of abuse in these areas. SPOT was used to image sections at specific Bregma points containing the regions of interest, which were saved as .TIFF files at 10x magnification. C-fos positive cells were easily visualized due to their dark staining, with the use of ImageJ software to automatically count cells (shown in Figure 5), The process of automated counting was adapted from a previously published method (Kholi PhD thesis) and actual counts and settings checked by a trained individual to ensure adequate detection of positive cells without interference from background noise.

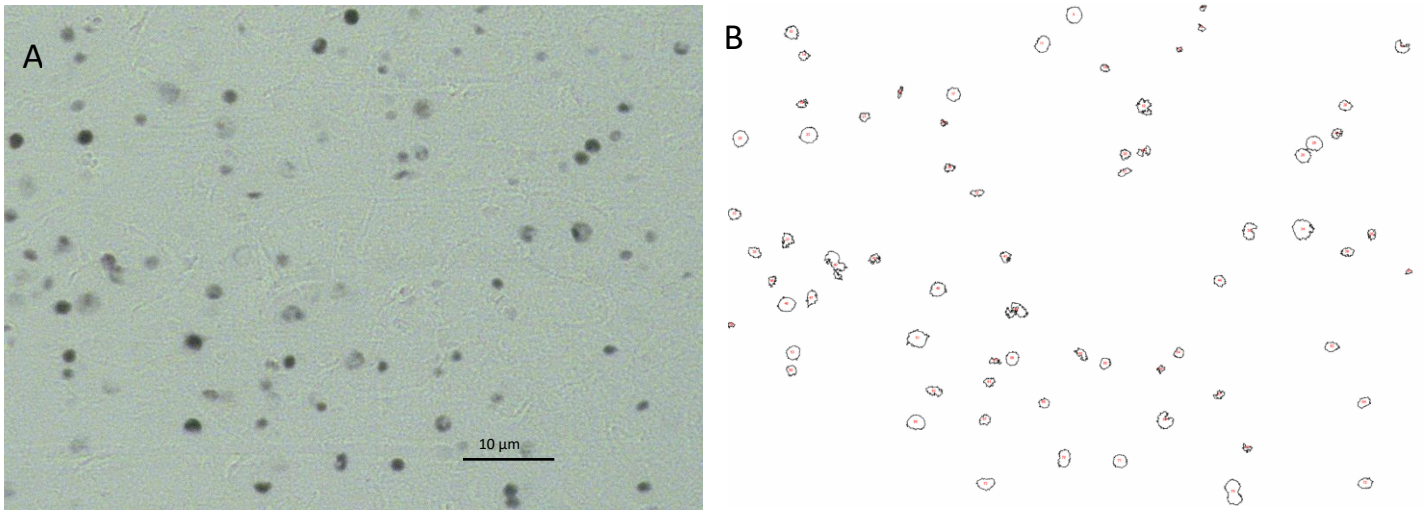


Figure 12. Example of the ImageJ counting process

A represents picture taken directly from the SPOT camera, B represents an example of the drawing ImageJ converts the image to for cell counting.

2.4 Statistical analysis

Prism GraphPad (v9) was used to analyse all statistical data, which was firstly checked for normality using the D'Agostino-Pearson normality test. Differences between groups were determined by a Two- way ANOVA, with the factor's caffeine and mephedrone to determine if there is an interaction between caffeine and mephedrone, and the factors WAY-100635 pre-treatment alongside combined caffeine plus mephedrone treatment to determine if the combined effect of caffeine and mephedrone is blocked by WAY-100635. This was followed by Tukey's post hoc test for multiple comparisons. The relevant post hoc tests are summarized in table 8. All data is expressed as mean ± sem and $p<0.05$ was considered to be significant.

Treatment combinations used in the post-hoc tests to identify the interaction between mephedrone and caffeine	**Treatment combinations used in the post-hoc tests to identify the impact of WAY-100635 on combination treatment**
Vehicle Vs Caffeine	WAY-100635 vs Vehicle
Vehicle Vs Mephedrone	Combination treatment vs Vehicle
Vehicle Vs Combination treatment	Combination treatment vs WAY-100635
Combination treatment vs Caffeine	
Combination treatment vs Mephedrone	

Table 8. Relevant treatment combinations used in the post hoc tests
Combination treatment refers to the co-administration of caffeine and mephedrone

Pearson's correlation analysis was used to determine positive associations between the change in c-fos count and magnitude of head weaving (per 5 min), locomotor activity (total distance moved; cm) or temperature change from baseline (°C) in each rat.

3.0 Results

3.1 Change in c-fos immunoreactivity following caffeine x mephedrone administration, and the effect of WAY-100635

C-fos is an important tool to identify activated neurons and can be used to identify regions in which respond to drugs of abuse such as mephedrone and caffeine. Using a 2-way ANOVA, we found a significantly higher density of cells expressing c-fos in the caffeine x mephedrone treatment groups in the pre-limbic cortex (Figure 21a), NacC (Figure 18c) and NacS (Figure 18d), and the ventral striatum (Figure 18). However, there was no effect seen of caffeine and mephedrone observed in the hypothalamus (Figure 20a) or dorsal striatum (Figure 18b). WAY-100635 did not influence the combined effect of mephedrone caffeine treatment on c-fos counts in any region. There was a main effect of caffeine + mephedrone, which was not affected by WAY in both the NacC ($F_{(1,23)} = 30.62$, $P<0.0001$) (Figure 25c) and the NacS ($F_{(1,23)} = 26.57$, $P<0.0001$) (Figure 25d).

In the pre-limbic cortex, there was a main effect of caffeine & mephedrone, and post-hoc tests revealed significant increase of 138% between vehicle + vehicle versus vehicle + caffeine & mephedrone + WAY-100635 (P=0.0004), 167% increase in vehicle + WAY versus caffeine & mephedrone + WAY (P=0.0011) and 161.9% increase in vehicle + WAY versus vehicle + Caffeine & Mephedrone (P=0.0015).

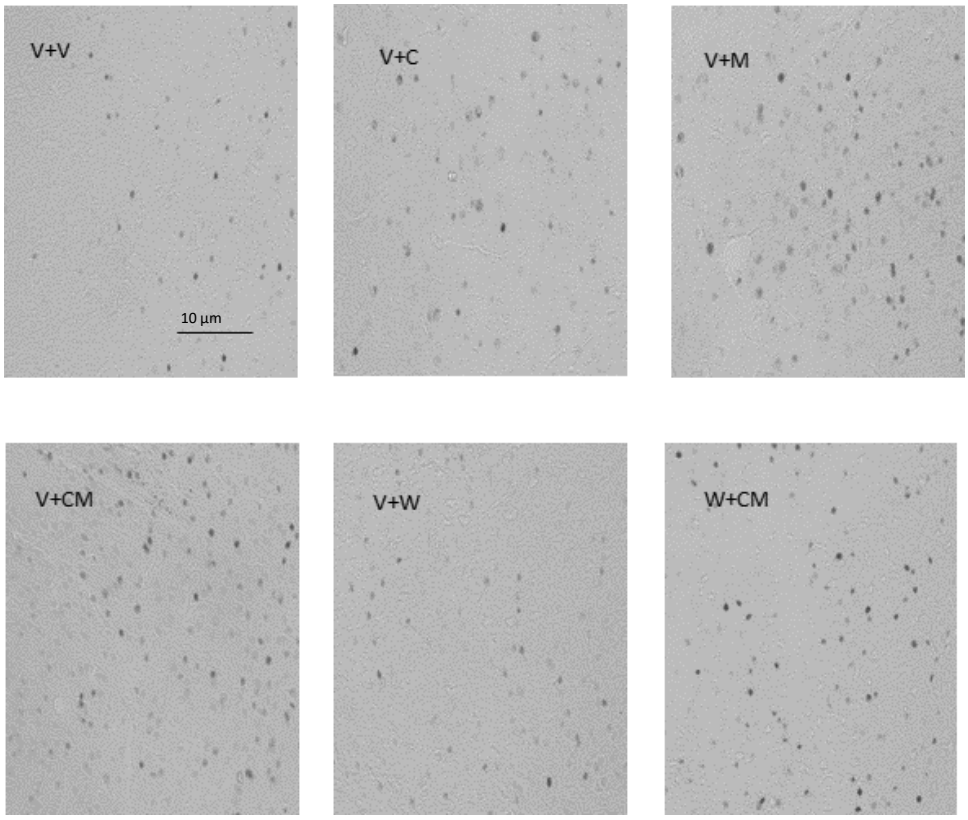


Figure 13. Representative pictures of the differing treatment groups in the pre-limbic cortex

(V, Vehicle; C, Caffeine; M, Mephedrone; W, Way-100635). Images that represent the group mean for the treatment groups in the prelimbic cortex

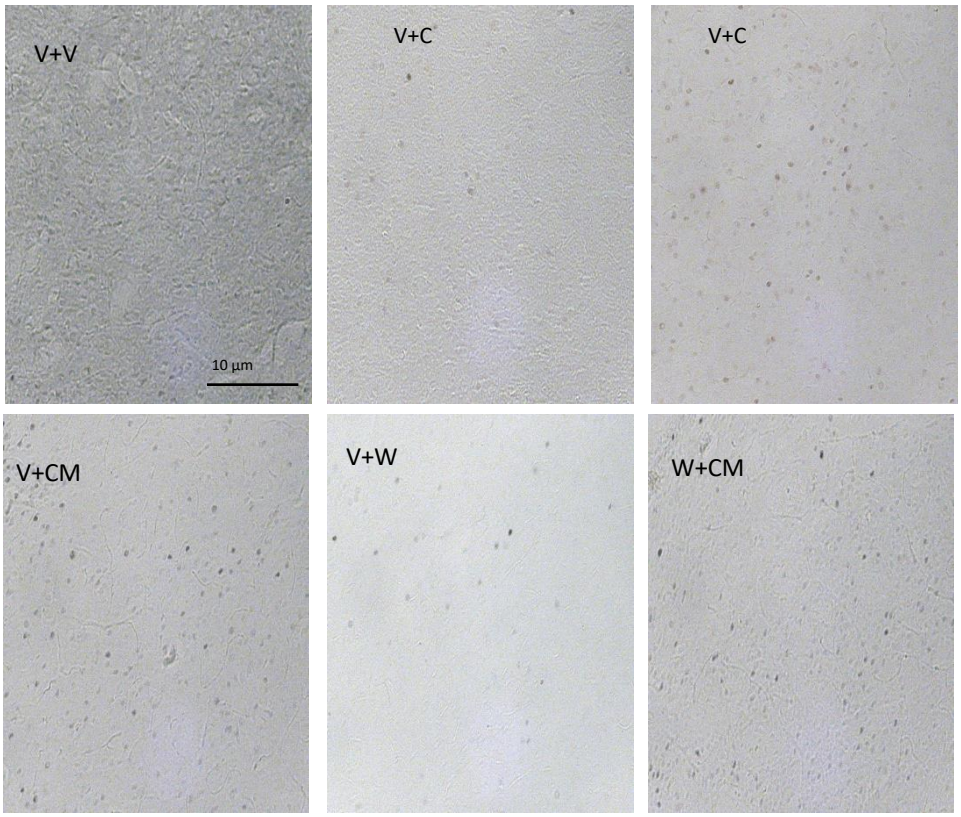


Figure 14. Representative pictures of the differing treatment groups in the NacC (V, Vehicle; C, Caffeine; M, Mephedrone; W, Way-100635). Images that represent the group mean for the treatment groups in the NacC.

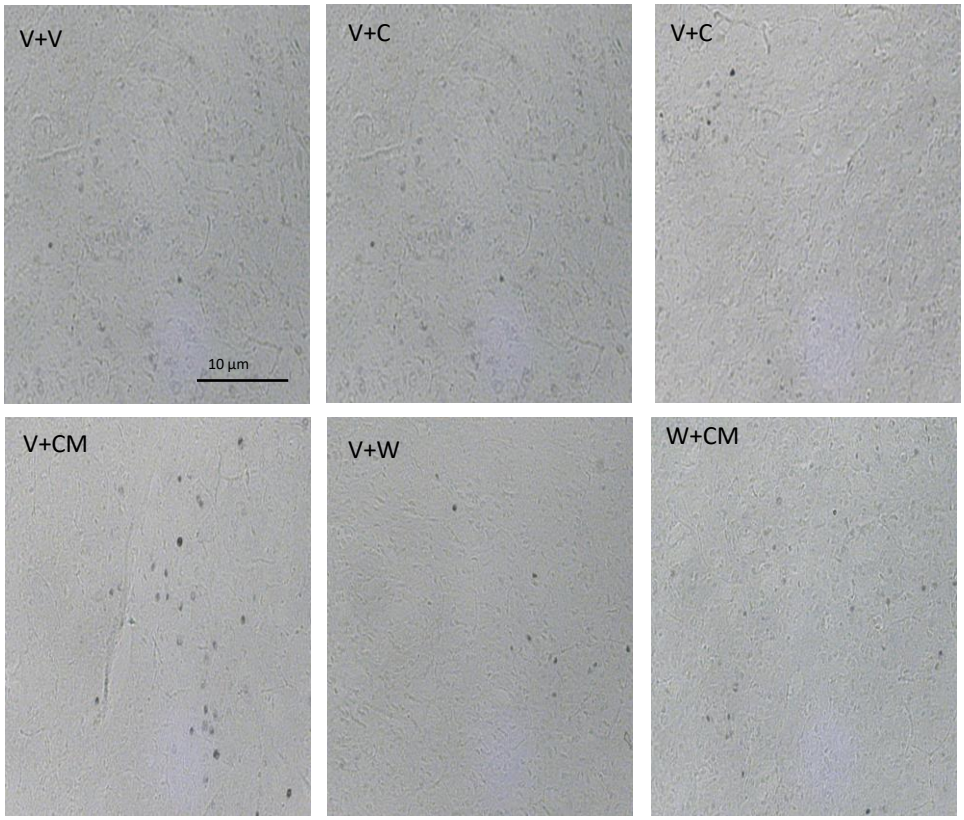


Figure 15. Representative pictures of the differing treatment groups in the NacS (V, Vehicle; C, Caffeine; M, Mephedrone; W, Way-100635). Images that represent the group mean for the treatment groups in the NacS.

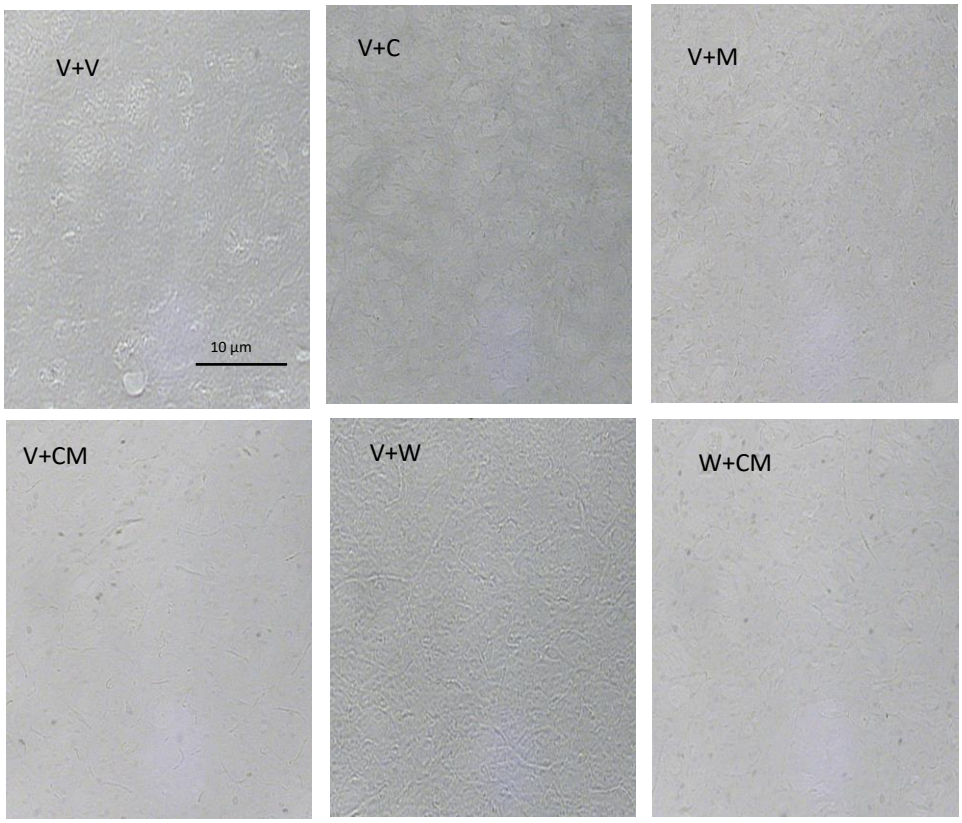


Figure 16. Representative images of the ventral striatum

(V, Vehicle; C, Caffeine; M, Mephedrone; W, Way-100635). Images that represent the group mean for the treatment groups in the ventral striatum

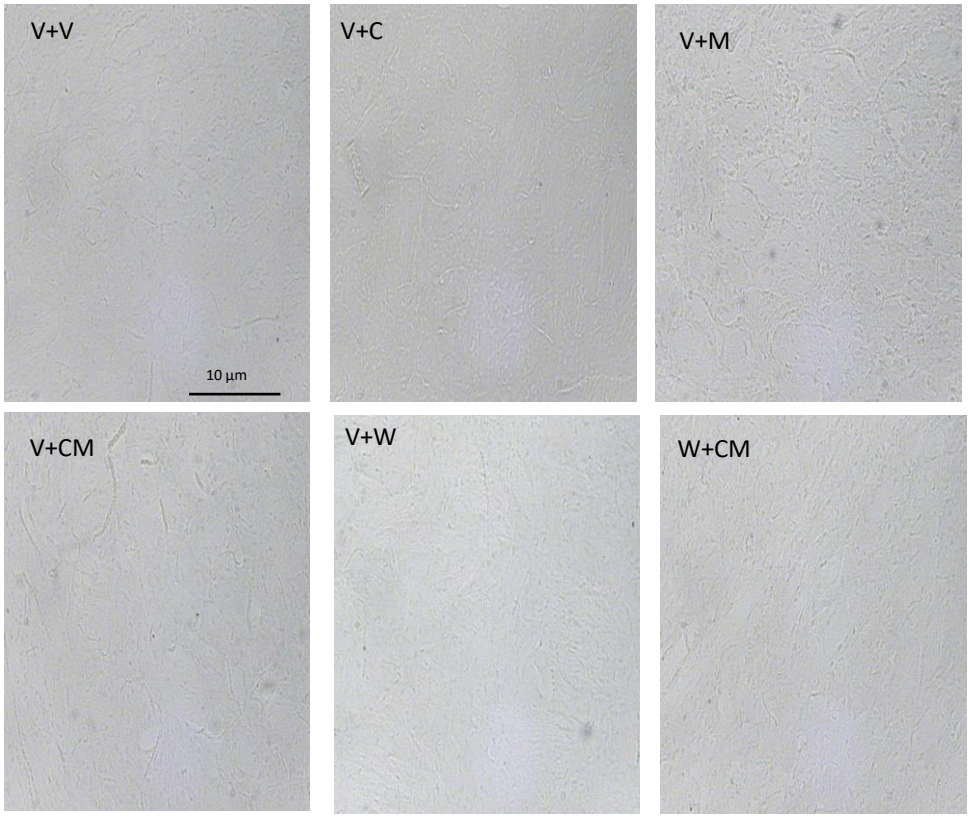


Figure 17. Representative images of the dorsal striatum

(V, Vehicle; C, Caffeine; M, Mephedrone; W, Way-100635). Images that represent the group mean for the treatment groups in the dorsal striatum.

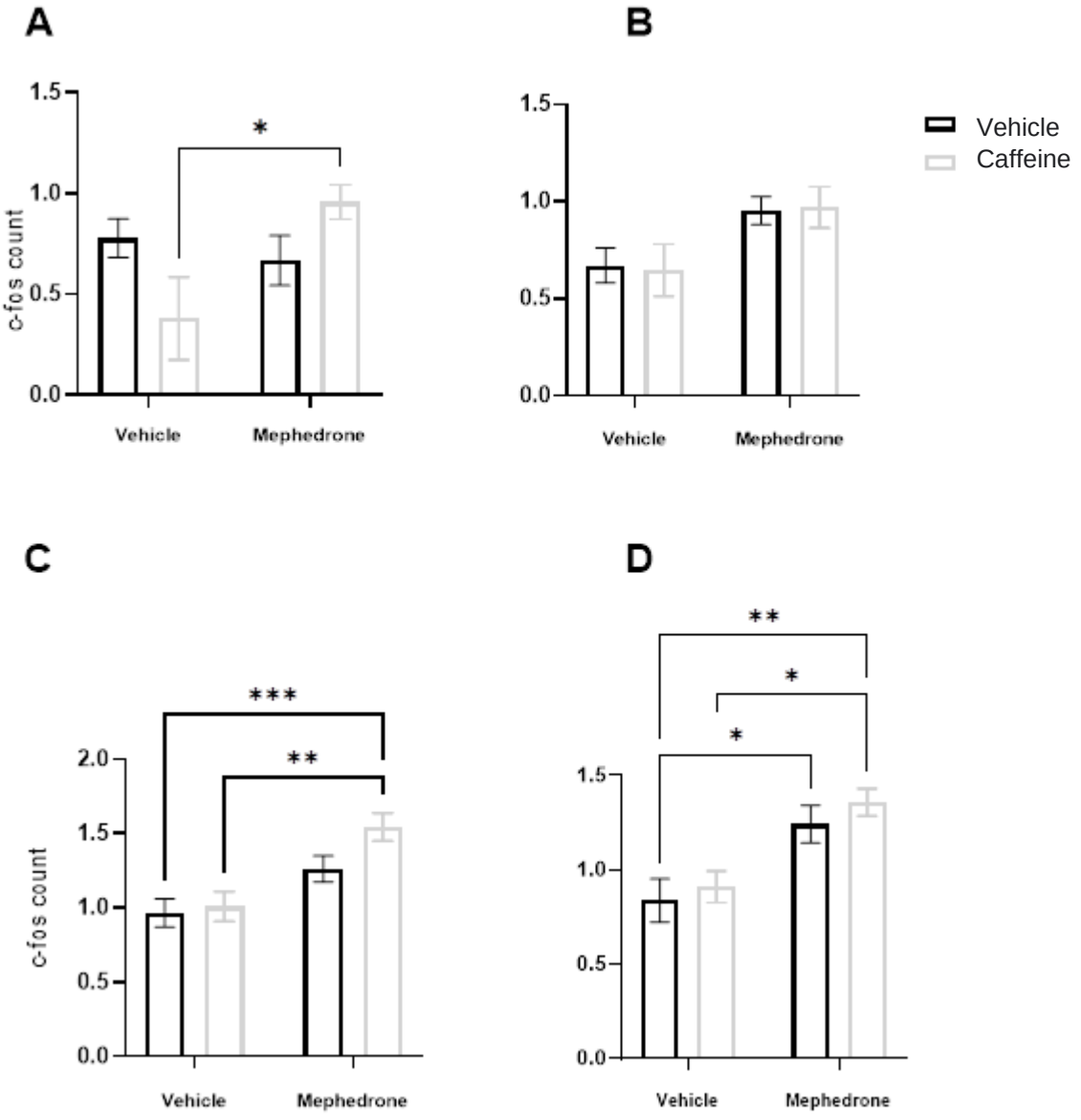


Figure 18. Effect of Mephedrone and caffeine administration on C-fos expression in the striatum

Bars represent differences (Mean ± SEM positive cell counts, n=8) of c-fos+ counts in the dorsal striatum (A), ventral striatum (B), NacC (C) and NacS (D) in singly-housed adult male Lister hooded rats that received i.p. pre-treatment with vehicle (saline, 1 mL kg^{-1}) or

mephedrone (10 mg kg^{-1}; Meph), at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), or caffeine plus mephedrone (10 mg kg^{-1} each combined in the same injection. (Raw data displayed on graphs, however this was not normally distributed so statistical analysis was completed with Log10 using Tukey's multiple comparison post-hoc following two-way repeated-measures ANOVA).

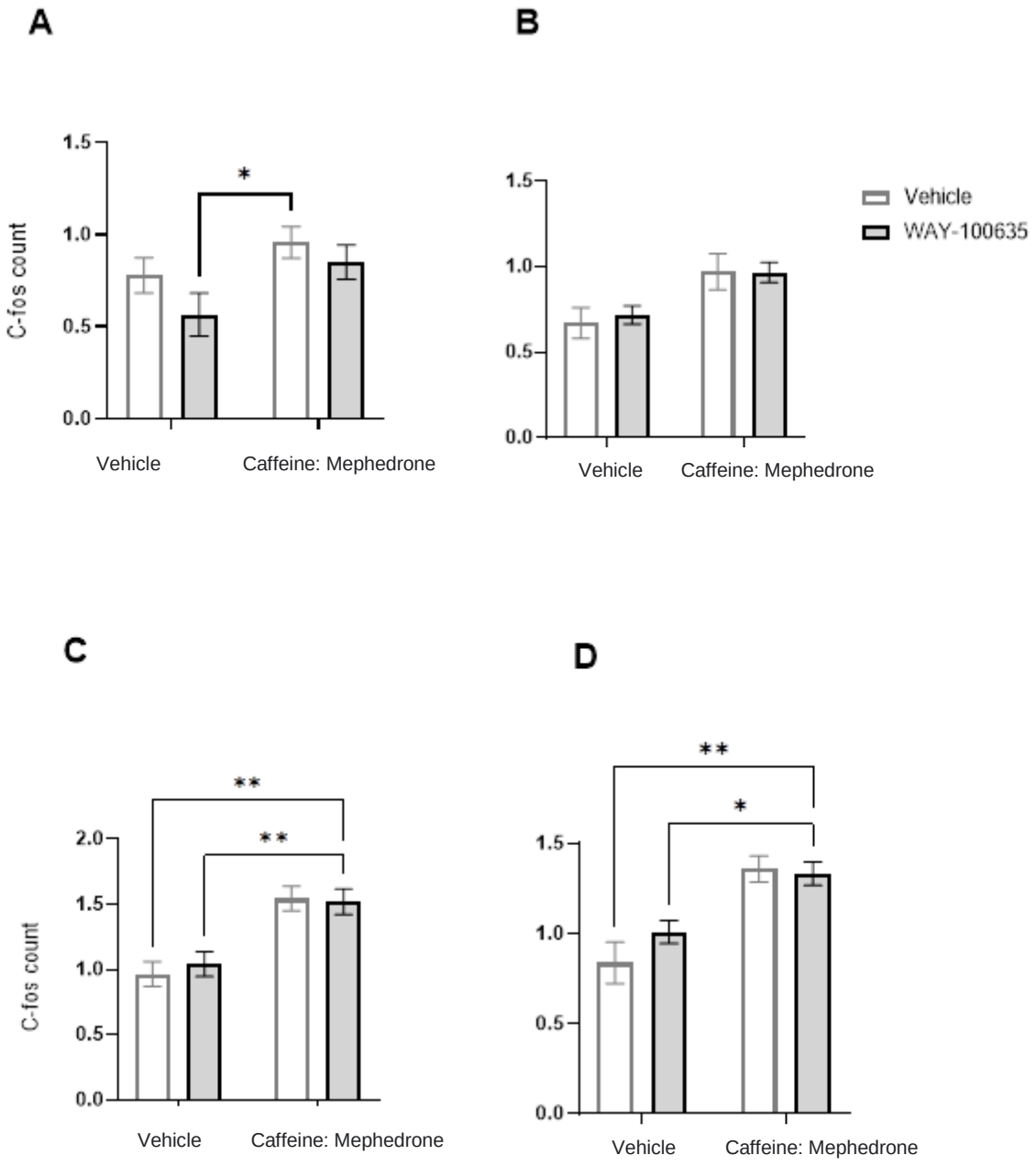


Figure 19. Effect of WAY-100635 on mephedrone x caffeine induced C-fos expression in the striatum.

Bars represent differences (Mean ± SEM, n=8) of C-fos+ counts in the dorsal striatum (A), ventral striatum (B), NacC (C) and NacS (D) in singly-housed adult male Lister hooded rats that received i.p. pre-treatment with vehicle (saline, 1 mL kg^{-1}) or WAY-100635 (0.5 mg kg^{-1}; WAY) at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), mephedrone (10 mg kg^{-1}; Meph), or caffeine plus mephedrone (10 mg kg^{-1} each combined in the same injection. (Raw data displayed on graphs, however this was not normally distributed so statistical analysis was completed with Log10 using Tukey's multiple comparison post-hoc following two-way repeated-measures ANOVA).

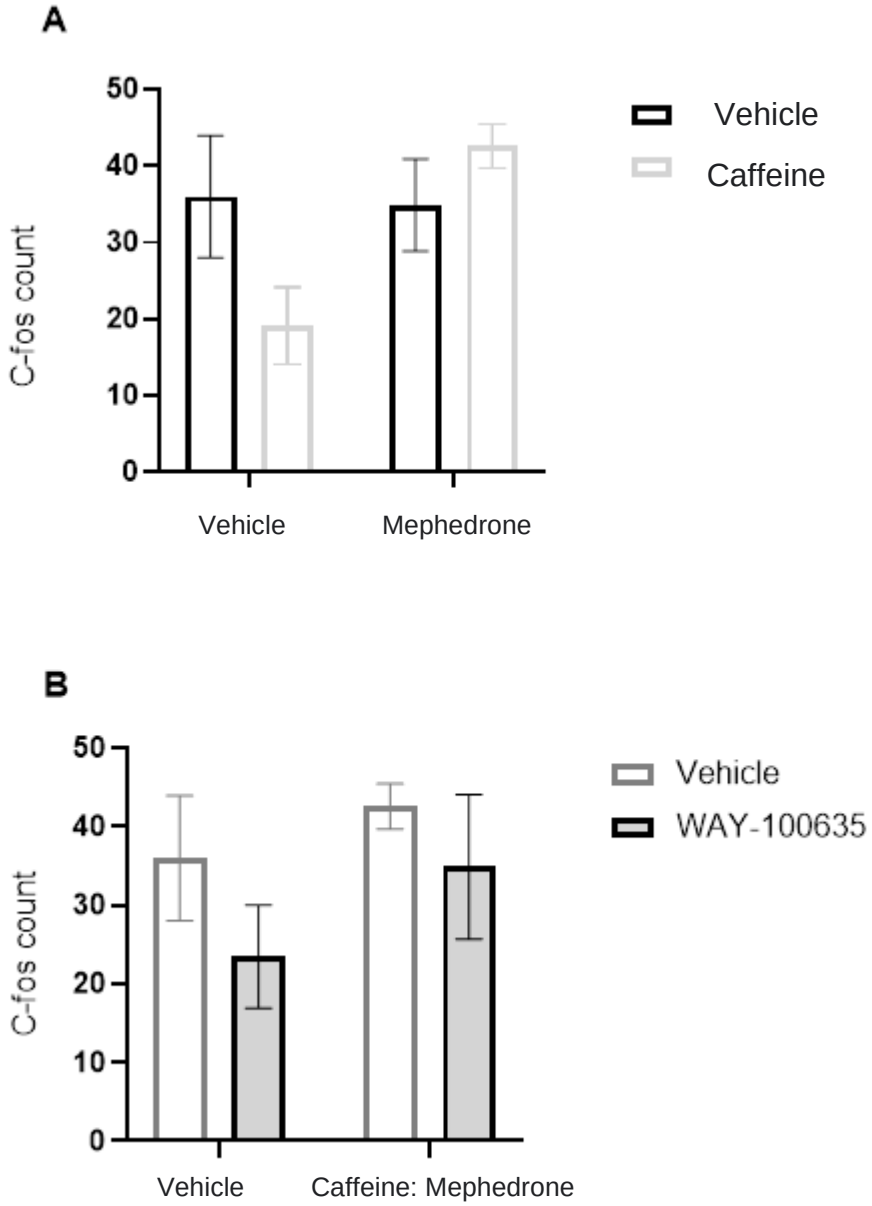


Figure 20. C-fos expression in response to (A) caffeine x mephedrone administration and (B) the effect of WAY-100635 on this expression in the hypothalamus

Number of C-fos+ counts (Mean ± SEM n=6) in the hypothalamus in singly-housed adult male Lister hooded rats that received i.p. pre-treatment with (A) vehicle (saline, 1 mL kg^{-1}) or mephedrone (10 mg kg^{-1}; Meph), at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), or caffeine plus mephedrone (10 mg kg^{-1} each combined in the same injection) or (B) vehicle (saline, 1 mL kg^{-1}) or WAY-100635 (0.5 mg kg^{-1}) at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg

kg-1), mephedrone (10 mg kg^{-1}), or caffeine plus mephedrone (Statistical analysis was completed using Tukey's multiple comparison post-hoc following two-way repeated-measures ANOVA).

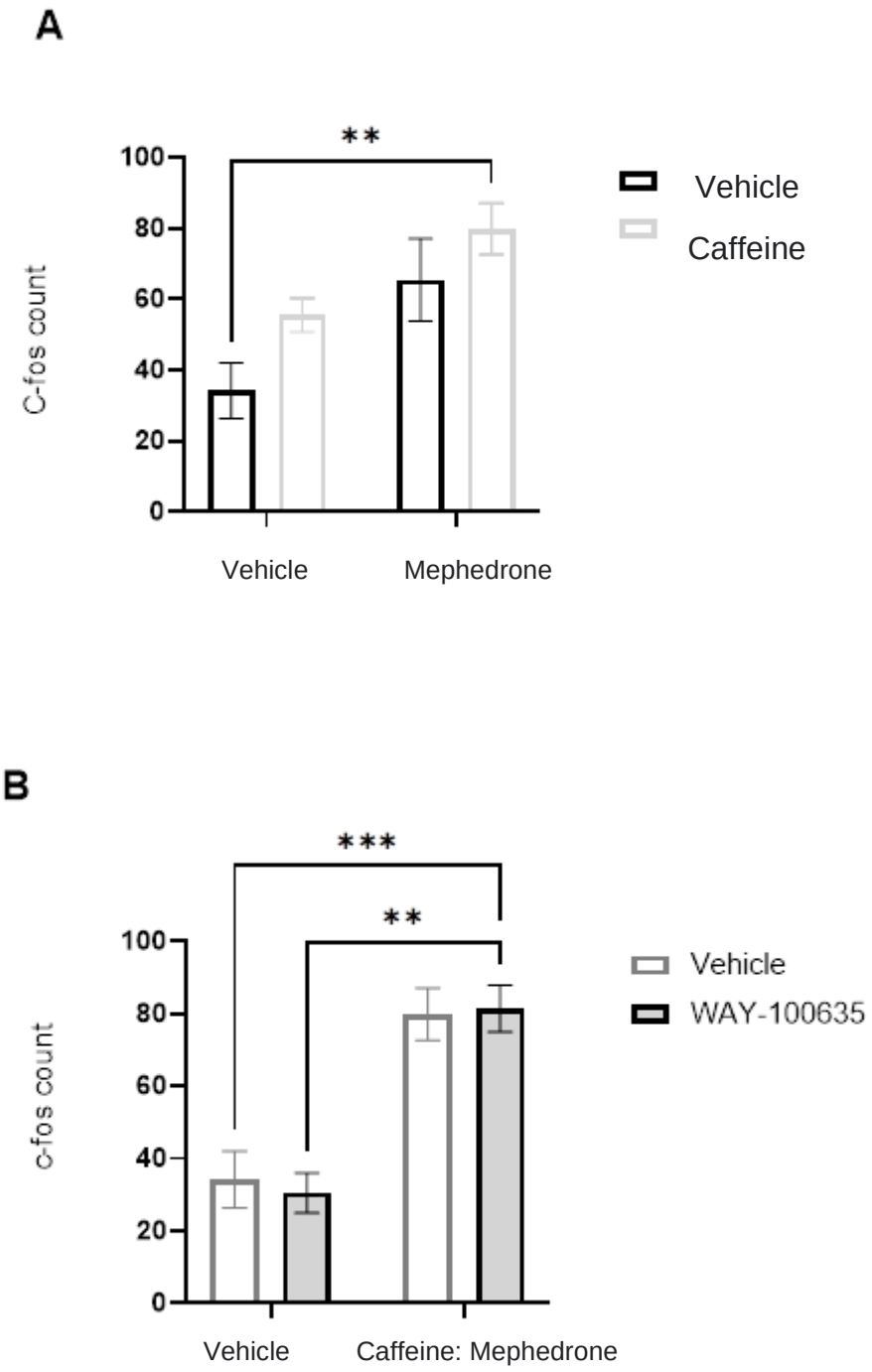


Figure 21. Alteration in c-fos expression in response to (A) caffeine x mephedrone administration and (B) the effect of WAY-100635 on this expression in the prelimbic cortex

Number of C-fos+ counts (Mean ± SEM, n=8) in the prelimbic cortex in singly housed adult male Lister hooded rats that received i.p. pre-treatment with either vehicle (saline, 1 mL kg^{-1}) or mephedrone (10 mg kg^{-1}; Meph), at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), or caffeine plus mephedrone (10 mg kg^{-1} each combined in the same injection) or vehicle (saline, 1 mL kg^{-1}) or WAY-100635 (0.5 mg kg^{-1}; WAY) at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), mephedrone (10 mg kg^{-1}; Meph), or caffeine plus mephedrone. WAY-100635 & Caff + Meph versus Vehicle & Vehicle. Tukey's multiple comparison post-hoc following two-way repeated measures ANOVA).

3.2 Correlation analysis

In order to see if the magnitude of c-fos change in expression was related to magnitude of any of the drug induced changes in behaviour or body temperature, the actual change in neurochemical count and behavioural measure was examined in each individual rat.

In the dorsal striatum (fig 28a,29a,30a), there was significant correlation of c-fos counts with locomotor activity (total distance moved; cm) (P= <0.001) and head weaving (per 5 min) (P= <0.001), but no significant correlation was found with temperature change from baseline (°C).

In both the ventral striatum (figure 28b,29b,30b) and the hypothalamus (figure 30), there were no significant correlations between the number of c-fos positive cells and the magnitude of either number of head weaving (per 5 min), locomotor activity (total distance moved; cm) or temperature change from baseline (°C).

In the NacC and NacS (figures 28c/d, 29c/d and 30c/d, respectively), a positive correlation was found between number of c-fos cells and the magnitude of head weaving (per 5 min) (P=<0.001), locomotor activity (total distance moved; cm) (P=<0.0001) and temperature change from baseline (°C) (P=<0.01 and P=<0.001, respectively) in all rats irrespective of the treatment they had received.

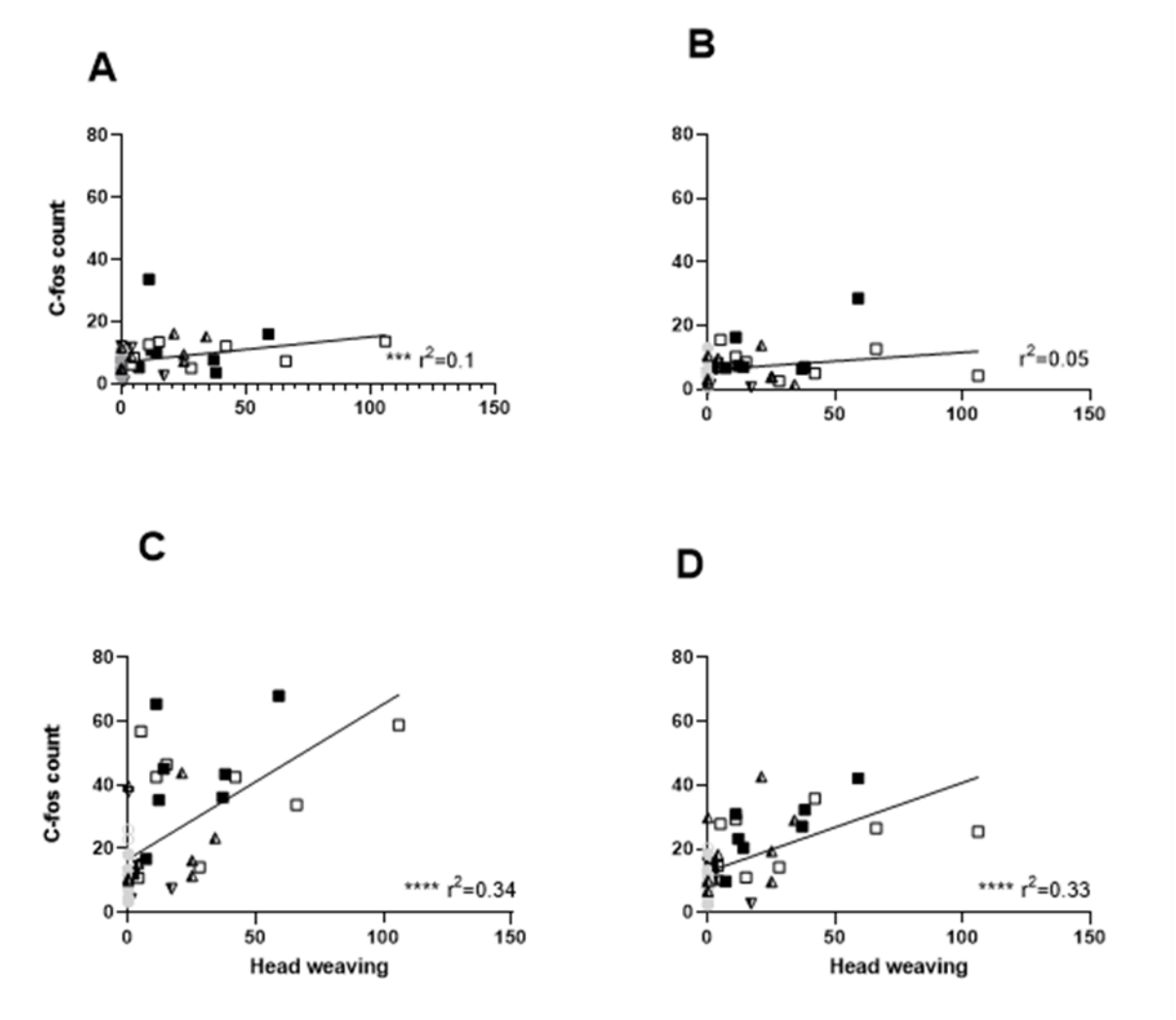


Figure 22. Effect of mephedrone and caffeine co-administration of head weaving behaviour

Linear correlation analysis of positive c-fos cells and head weaving counts in the striatum in singly-housed adult male Lister hooded rats that received i.p. pre-treatment with (A) vehicle (saline, 1 mL kg^{-1}) or mephedrone (10 mg kg^{-1}; Meph), at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), or caffeine plus mephedrone

(10 mg kg^{-1} each combined in the same injection) or (B) vehicle (saline, 1 mL kg^{-1}) or WAY-100635 (0.5 mg kg^{-1}) at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}), mephedrone (10 mg kg^{-1}), or caffeine plus mephedrone #P<0.05 Vehicle & Caff + Mephedrone versus Vehicle & Vehicle; $P<0.05 WAY-100635 & Caff + Mephedrone versus WAY-100635 & Vehicle. C-fos counts are positively correlated with head weaving, with significance reached in the dorsal striatum (A), both the NacC (C), and NacS (D), but not in the ventral striatum (B). ****P<0.0001; ***P<0.001.

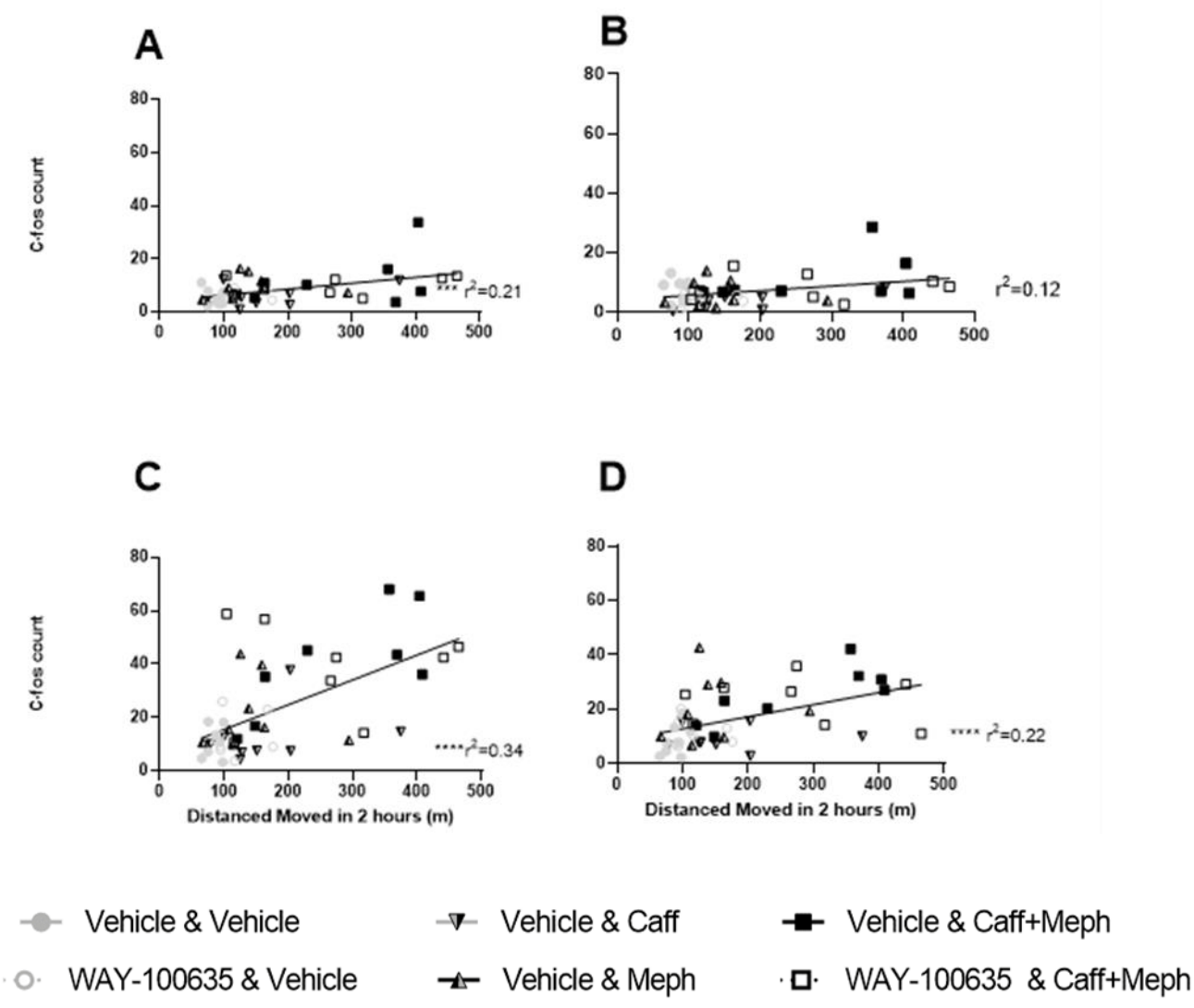


Figure 23. The effect of mephedrone and caffeine on locomotor behaviour

Correlation analysis of positive c-fos cells and distance moved in 2 hours in the striatum in

singly-housed adult male Lister hooded rats that received i.p. pre-treatment with (A) vehicle (saline, 1 mL kg^{-1}) or mephedrone (10 mg kg^{-1}; Meph), at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), or caffeine plus mephedrone (10 mg kg^{-1} each combined in the same injection) or (B) vehicle (saline, 1 mL kg^{-1}) or WAY-100635 (0.5 mg kg^{-1}) at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}), mephedrone (10 mg kg^{-1}), or caffeine plus mephedrone #P<0.05 Vehicle & Caff + Mephedrone versus Vehicle & Vehicle; $P<0.05 WAY-100635 & Caff + Mephedrone versus WAY-100635 & Vehicle. C-fos counts are positively correlated with locomotor activity, with significance reached in the dorsal striatum (A), both the NacC (C), and NacS(D), but not in the ventral striatum (B) (Statistical analysis completed with Pearson's correlation). ****P<0.0001; ***P<0.001

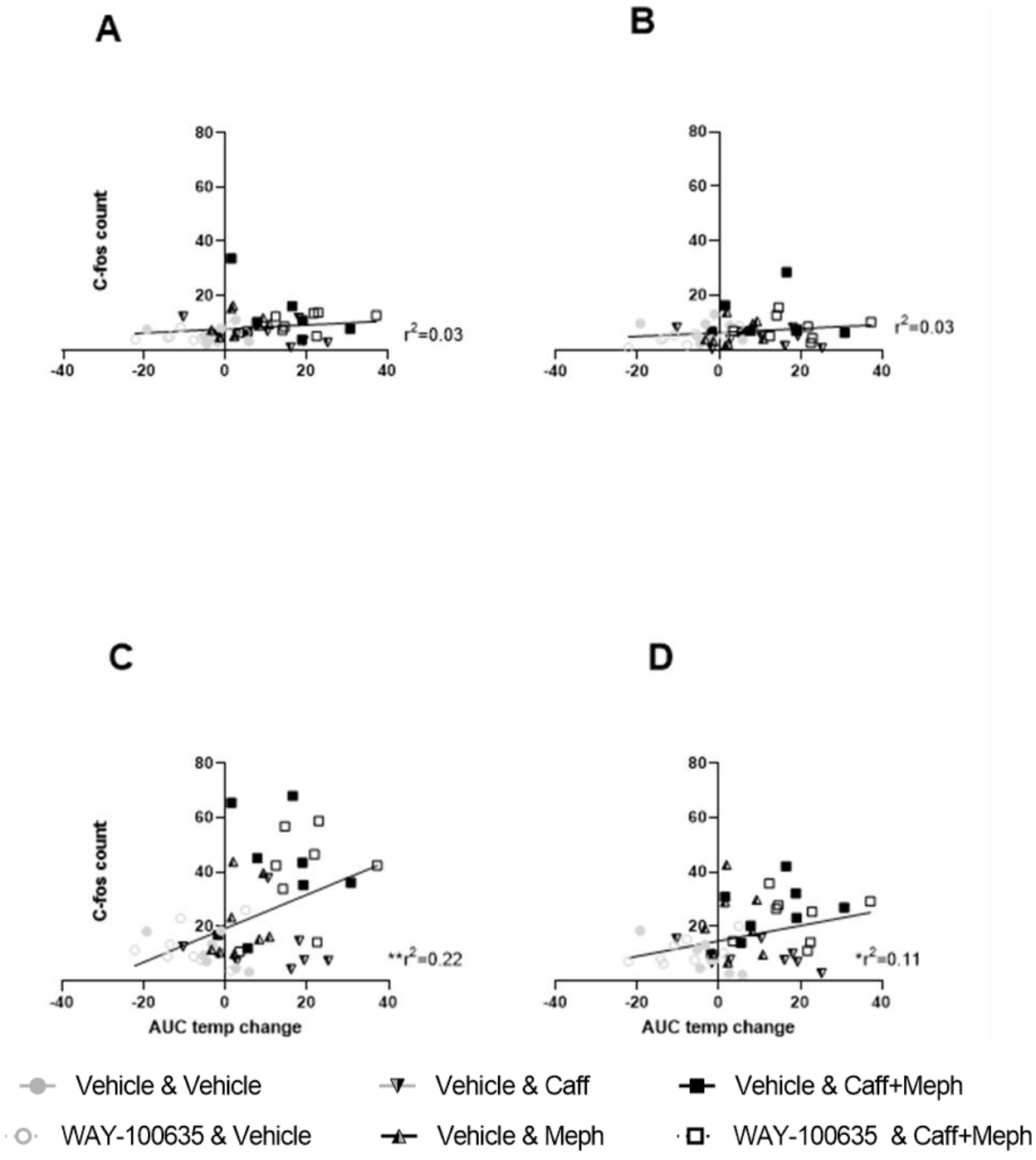


Figure 24. The effect of mephedrone and caffeine on body temperature

Correlation analysis of positive c-fos cells and temperature change in 2 hours in the striatum. hypothalamus in singly-housed adult male Lister hooded rats that received i.p. pre-treatment with (A) vehicle (saline, 1 mL kg^{-1}) or mephedrone (10 mg kg^{-1}; Meph), at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), or caffeine plus mephedrone (10 mg kg-1 each combined in the same injection) or (B) vehicle (saline, 1 mL

kg^{-1}) or WAY-100635 (0.5 mg kg^{-1}) at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}), mephedrone (10 mg kg^{-1}), or caffeine plus mephedrone #P<0.05 Vehicle & Caff + Mephedrone versus Vehicle & Vehicle; $P<0.05 WAY-100635 & Caff + Mephedrone versus WAY-100635 & Vehicle-fos counts are positively correlated with temperature change in the NacC (C) and NacS (D), but not in the ventral (A) or dorsal striatum (B). *P<0.1; **P<0.01

Statistical analysis completed with Pearson's correlation.

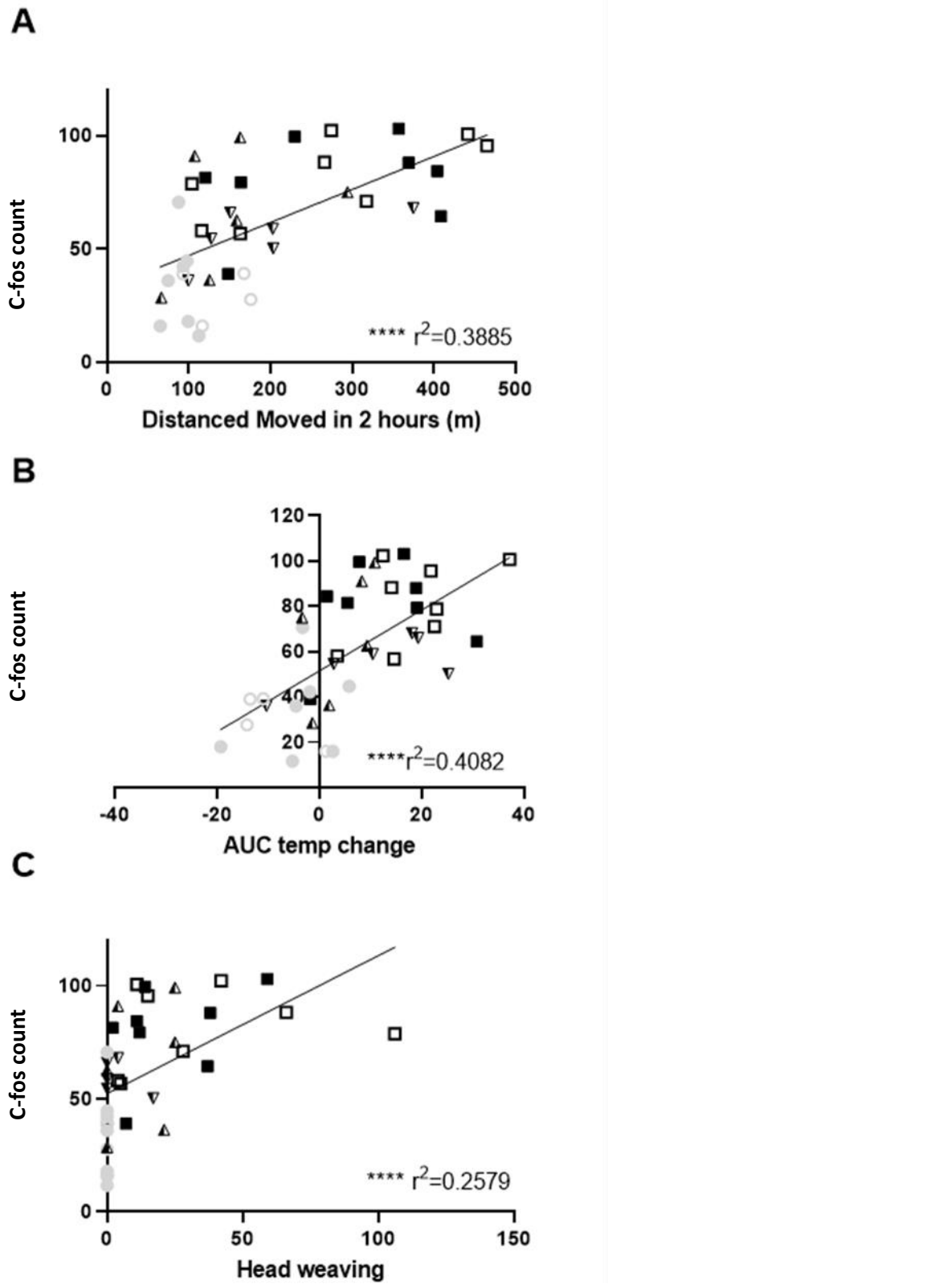

A
C-fos count
100
50
0
0
100
200
300
400
500
Distanced Moved in 2 hours (m)
**** r2=0.3885
B
C-fos count
120
100
80
60
40
20
-40
-20
0
20
40
AUC temp change
****r2=0.4082
C
C-fos count
100
50
0
0
50
100
150
Head weaving
**** r2=0.2579
Vehicle & Vehicle
Vehicle & Caff
Vehicle & Caff+Meph
WAY-100635 & Vehicle
Vehicle & Meph
WAY-100635 & Caff+Meph

Figure 25. The effect of Mephedrone x Caffeine administration in the pre-limbic cortex on (A) locomotor activity, (B) Temperature change and (C) head weaving

Correlation analysis of positive c-fos cells and (a) locomotor activity, (B) temperature change and (C), head weaving in the prelimbic cortex, in singly-housed adult male Lister hooded rats that received i.p. pre-treatment with (A) vehicle (saline, 1 mL kg^{-1}) or mephedrone (10 mg kg^{-1}; Meph), at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), or caffeine plus mephedrone (10 mg kg^{-1} each combined in the same injection) or (B) vehicle (saline, 1 mL kg^{-1}) or WAY-100635 (0.5 mg kg^{-1}) at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}), mephedrone (10 mg kg^{-1}), or caffeine plus mephedrone. ****P<0.0001.

Statistical analysis completed with Pearson's correlation.

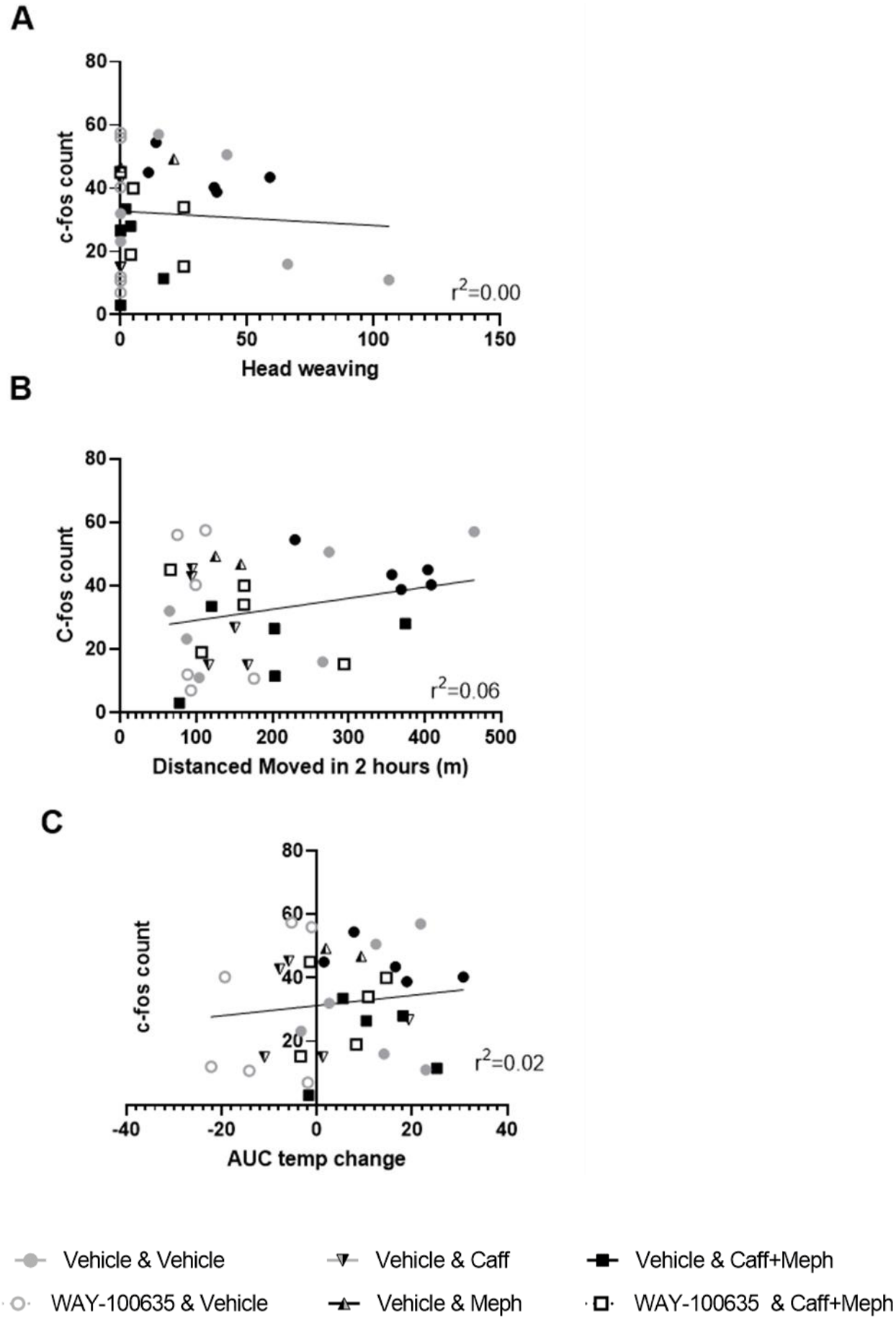

A
c-fos count
Head weaving
r2=0.00
B
C-fos count
Distanced Moved in 2 hours (m)
r2=0.06
C
c-fos count
AUC temp change
r2=0.02
Vehicle & Vehicle
Vehicle & Caff
Vehicle & Caff+Meph
WAY-100635 & Vehicle
Vehicle & Meph
WAY-100635 & Caff+Meph

Figure 26. The effect of Mephedrone x Caffeine administration in the hypothalamus on (A) locomotor activity, (B) Temperature change and (C) head weaving

Correlation analysis of positive c-fos cells and (a) locomotor activity, (B) temperature change and (C), head weaving, in the hypothalamus in singly-housed adult male Lister hooded rats that received i.p. pre-treatment with (A) vehicle (saline, 1 mL kg^{-1}) or mephedrone (10 mg kg^{-1}; Meph), at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}; Caff), or caffeine plus mephedrone (10 mg kg^{-1} each combined in the same injection) or (B) vehicle (saline, 1 mL kg^{-1}) or WAY-100635 (0.5 mg kg^{-1};) at -30 min, followed by i.p. treatment with vehicle (saline, 1 mL kg^{-1}), caffeine (10 mg kg^{-1}), mephedrone (10 mg kg^{-1}), or caffeine plus mephedrone. C-fos counts did not display any significant correlations with locomotor activity (A), temperature change (B) or head weaving (C). Statistical analysis completed with Pearson's correlation.

4.0 Discussion

The main findings of this study are (1) mephedrone alone increases c-fos expression in the NacS (2) caffeine co-administration with mephedrone increases c-fos expression above control while neither caffeine nor mephedrone alone have any significant effect in both the NacC and NacS, alongside the ventral striatum and the prelimbic cortex, (3) inhibition of the 5-HT_{1A} receptor by WAY-100635 did not have an impact on mephedrone and caffeine induced c-fos expression nor influence any of the behavioural or physiological measures studied and (4) mephedrone-induced locomotion and head weaving displayed a significant correlation with c-fos expression in the dorsal striatum, and both the NacC and NacS. Temperature change displayed a positive correlation with the NacS and NacC. The present study also complements the existing literature surrounding the psychoactive effects of mephedrone, confirming that it induces profound locomotor activity and body temperature changes in rats, which are influenced by caffeine co-administration. The results also show that caffeine increases mephedrone induced c-fos expression in the regions examined, which is in line with the behavioural studies performed, which found combination treatment displayed an additive effect via increasing locomotion, temperature, and stereotyped behaviour, in which WAY-100635 had no effect. Although WAY-100635 was expected to attenuate c-fos expression throughout the brain regions examined, the lack of inhibition correlates well with the lack of effect of WAY-100635 on the behavioural and physiological responses (observed in the previous study performed by a PhD student, see section 1.3). Both the c-fos expression and behavioural responses were expected to be influenced by this inhibition of the 5-HT_{1A} receptor induced by combination treatment, as it has previously been reported that pre-treatment with WAY-100635 abolished mephedrone induced hypothermia (Shortall et al, 2015).

Region	Vehicle	Mephedrone	Caffeine	Mephedrone + Caffeine	WAY-100635
Dorsal Striatum	-	-	-	-	-
Ventral Striatum	-	-	-	↑	-
Nucleus Accumbens Core	-	-	-	↑	-
Nucleus Accumbens Shell	-	↑	-	↑	-
Pre-limbic cortex	-	-	-	↑	-
Hypothalamus	-	-	-	-	-

Table 7. The effect of each treatment on c-fos expression

Results from the post-hoc test

↑ indicates increase in c-fos expression; - indicates no significant change in c-fos expression

4.1 NacC and NacS

Within the Nac, mephedrone induced c-fos expression within the NacS, but not the NacC. Although mephedrone was hypothesised to increase c-fos expression throughout the Nac, only a significant expression of c-fos was found in the NacS. Given that there are clear differences (see section 1.1.4.3), this is not entirely unsurprising, though still of interest. The mephedrone induced c-fos expression that occurred selectively within the NacS is in agreement with similar previous rat studies (Motbey et al, 2011) and other studies who found psychostimulants affect these regions differently, as significant c-fos expression has been found in the Nac in response to amphetamine (Lin et al, 1996); Johansson, Lindström and Fredholm, 1994); Colussi-Mas et al, 2007), cocaine (Marie-Claire et al, 2003); Lucas, Segu and Hen, 1996; Johansson et al, 1994; Torres and River, 1994), methamphetamine (Thiriet, Zwiller Ali, 2001; Umino, Nishikawa and Takashi, 1995) and MDMA (Stephenson, Hunt, Topple and McGregor, 1999; Marie-Claire et al, 2003). However, previous groups have

reported expression within the NacC, but not the NacS, following methamphetamine administration (Cornish et al, 2012; Rossi et al, 2020), which highlights the differences between mephedrone and its related compounds. Alongside this, the varied c-fos expression of these regions further supports previous literature suggesting the NacC and NacS mediate different and specific functions, giving further insight to how mephedrone exerts its effects.

In response to caffeine alone, there was no increase in c-fos expression. This was expected, as previous research has found caffeine only induces c-fos expression following much larger doses, such as 75mg/kg (male Wistar rats, i.p.) or more (Bennett and Semba, 1998), and the current study, used a much lower dose (10mg/kg). However, when co-administered with mephedrone, caffeine induced an increase in c-fos expression in both the NacC and NacS. The increase in c-fos expression following combined treatment, compared to mephedrone alone was expected, as the current study (**in the previous work performed by a Ph.D. student**) found mephedrone (10 mg/kg, i.p.) co-administered with caffeine (10 mg/kg i.p.) enhanced locomotion and stereotyped behaviours in Lister hooded rats possibly by induction of a mild serotonergic syndrome and converts mephedrone induced hypothermia to hyperthermia. Indeed a further study in our laboratory (Shortall et al, 2016), also found the combined mephedrone and caffeine treatment attenuated mephedrone-induced anxiogenic behaviours. In addition, McNamara et al (2006), reported a clear additive effect when MDMA (15mg/kg; i.p.) was co-administered with caffeine (10 mg/kg; i.p.) in male Sprague-Dawley rats, converting the hypothermic response seen with MDMA alone, to lethal hyperthermia (at both a dose of 5mg/kg and 10mg/kg, but not 1mg/kg; i.p.). The additive effect that caffeine has on psychostimulants has been further reported with other co-administration studies using rodents, including cocaine (Bedingfeild, King and Holloway, 1998; Kuzmin et al, 1999; Derlet et al, 1992) and amphetamine (Derlet et al, 1992)

methamphetamine (Sinchai et al, 2011; Kuribara, 1994), which in conjunction with the current results, suggest caffeine has the potential to exacerbate the adverse effects of psychostimulants.

Caffeine (at doses 10mg/kg and 30mg/kg, but not 3 or 100 mg/kg) has been shown to increase dopamine transmission in the NacS, which is also seen with selective A_1 antagonism (via 8-cyclopentyltheophylline), but not with selective A_2 antagonism (via SCH 58261) (Solinas et al, 2002), which suggests the modulatory effect of caffeine in the NacS is due to the inhibition of A_1 receptors, and justifies the expression seen only with combination treatment. This is further supported by a study using genetically modified mice, in which it was reported that the reinforcing effects of MDMA were attenuated in A_{2A} knockout mice, which was seen by the absence of self-administration in these mice (Ruiz-Medina et al, 2011). This further implicates caffeine in the modulation of reinforcement of psychostimulants and suggests that it is the blockade of the A_1, but not the A_2 receptor that is important for the increased c-fos expression, and increased rewarding effects that caffeine induces.

4.1.1 Correlations observed in the Nucleus accumbens

The current study also observed correlations with the both the NacC and NacS and locomotion. Previous studies have implicated the Nac in mediating the effects induced by psychostimulants (Moghaddam and Bunney, 1989). In agreement with the current results, Colussi-Mas and Schek (2008) reported a significant correlation between locomotion and c-fos positive neurons within the Nac following MDMA administration (10mg/kg i.p.). There is much evidence which links the Nac to psychostimulant induced locomotion (Boye, Grant and

Clarke, 2000), so this result was expected. For example, methylphenidate, a psychostimulant used to treat ADHD, induces locomotion which is profoundly increased in rats with lesions to the Nac (Podet et al, 2010). Specifically, many studies have demonstrated the importance of the dopaminergic system within the Nac in mediating psychostimulant induced locomotion (Kelly, Severe and Iverson, 1975; Roberts et al, 2012; Swanson et al, 1997; Gong et al, 1999; Dreher and Jackson,1989). Lesions of the DA system by 6-hydroxydopamine (8ug) in the Nac attenuates amphetamine induced locomotion (Kelly, Severe and Iversen, 1975), whereas direct administration of DA into the Nac exacerbates locomotion (Pijnenburg and Rossum, 1973). Cocaine, but not procaine and lidocaine (which both have low affinity for the dopamine transporter) injected into the Nac of rats produced a dose-dependent increase in locomotion, an effect which was blocked by cis-flupentixol, a DA receptor antagonist (Delfs, Schriber and Kelley, 1990). As mephedrone (3mg/kg s.c.) has been shown to increase DA in the Nac (Kehr et al, 2011), alongside the positive correlations seen in the current study, mephedrone induced DA release in the Nac is most likely the mechanism in which mephedrone induces an increased locomotion in rats, and may also explain the profound c-fos expression within this region. Specifically, research has reported that activation of D_1 neurons within the Nac results in both increased running and locomotion, whilst activation of D_2 neurons displayed an opposing effect (Zhu, Ottenheimer and DiLeone, 2016), therefore, it is likely that mephedrone acts upon the D_1 receptors in the Nac producing enhanced locomotion, resulting in increased c-fos expression as seen in the current study. In support of this, the increase in extracellular DA within the Nac correlates with locomotion induced by cocaine (Kalivas and Duffy, 1990). However, it is important to note this study used mice, and so the results should be taken with caution when associated with the results of the current study.

The current literature has also provided further evidence towards the NacC and NacS displaying differing functions, which can help to explain the difference in c-fos positive cells within these subregions in the current study. For example, A_{2A} antagonist (MSX-3) administered into the NacC, but not the NacS, reverses the inhibition of locomotion induced by administration of a dopamine antagonist (Haloperidol) (Salamone et al, 2008). Furthermore, the non-selective DA agonist SKF 38393 administered into the NacS, but not the NacC, increased locomotion and stereotyped behaviour (Ikemoto, 2002). In addition, lesions to the striatum (but not the Nac) attenuated PCP (phenyl cyclohexyl piperidine) induced stereotyped behaviour (Nabeshima et al, 1983), so the notion that the Nac is involved in drug induced stereotypy is controversial. However, in line with our results, Baumann, Clark and Rothman (2008), reported a dose-dependent increase in 5-HT in response to MDMA, which was found to be positively correlated with stereotyped behaviour in all of these three regions, in alignment with the current correlation with stereotypy in these regions. The conflicting results are most likely due to the multiple monoamine systems that MDMA and mephedrone influence, whereas administering a selective DA agonist may specifically alter DA system, and PCP (a non-competitive NMDA antagonist) has a distinct pharmacology from amphetamine-like stimulants. In further support of the results, Kelly, Seviour and Iveren (1975) reported that lesions to the caudate via 6-hydroxydopamine attenuated stereotype behaviour induced by amphetamine (5mg/kg), suggesting it is the DA system within the dorsal striatum that mediates stereotype behaviour. However, it is established that the serotonin system is vital for these stereotyped responses, and as mephedrone displays a high affinity for certain 5-HT receptors, this may contribute to the stereotyped behaviours observed.

C-fos expression in both the NacC and NacS also displayed a correlation with temperature change. Although not usually associated with thermoregulation, previous literature has shown that the Nac contains temperature sensitive neurons (Bachtell, Tsivkovskaia and Ryabinin, 2003). In addition, the dopaminergic system has been implicated in the control of thermoregulation, and as the Nac is primarily innovated with dopaminergic projections, this may explain these correlations. For example, injection of DA produces hypothermia at a normal room temperature and specifically, lesions to the Nac reduced amphetamine hyperthermia in rats (Wirtshafter, Asin and Kent, 1978). The same study also reported that these lesions did not influence hyperactivity, consistent with the idea that distinct regions are involved in these responses. However, without the use of microdialysis in the current study, it is impossible to know whether these correlations are dopamine dependent, or controlled by another neurotransmitter system, such as 5-HT.

4.2 Ventral and Dorsal Striatum

In response to mephedrone, no significant change in c-fos expression was observed in the dorsal striatum, which was surprising. As previously mentioned, (see 1.1.4.3) the dorsal striatum contains the Cp. Previous studies have observed c-fos expression in the caudate putamen following other stimulants such as cocaine (Lucas, Segu and Hen, 1996; Johansson et al, 1994; Marie-Claire et al, 2003; Torres and River, 1994), amphetamine (Lin et al, 1996; Johansson, Lindström and Fredholm, 1994), methamphetamine (Cornish et al, 2012; Thiriet, Zwiller Ali, 2001) and MDMA (Stephenson, Hunt, Topple and McGregor, 1999; Marie-Claire et al, 2003). Specifically, the dorsal striatum has been implicated in mediating goal-directed behaviour and stimulus driven responses (Hong et al, 2020), and can be further divided into two separate subregions; the dorsal lateral, and the dorsal medial striatum

(Lovinger and Mathur, 2016). These sub regions have been shown to mediate different responses, such that lesions to the dorsolateral striatum inhibit the formation of habits, an important evolutionary advantage to reduce the time of the brain spent for decision making. Lesions to the dorsal medial striatum have been shown to result in a reduction of goal directed behaviours (Burton, Nakamura and Roesch, 2015), which unlike habits, are purposeful and are based upon future consequences that produce beneficial rewards. Both habit behaviours, and goal directed behaviours can become maladaptive, and result in the onset of addictive behaviours. For example, addicts may continue to administer of recreational drugs even if the user is aware of negative consequences attached to drug seeking and taking, and this habit may be a consequence of a dysfunction in the region that controls goal-directed behaviours. If mephedrone were addictive it might be expected to activate neurons in the dorsal striatum, within this region, as alterations within this caused by psychostimulants may be responsible for the conversion of beneficial goal directed and habitual responses, to compulsive drug use.

The involvement of the ventral striatum in psychostimulant induced reward and subsequent addiction is also well documented (Shin, Liu and Ikemoto, 2008; Shin et al, 2009) and it was therefore surprising that no significant c-fos expression was observed in this region following mephedrone. The ventral striatum includes the Nac and striatal part of the olfactory tubercle, alongside the ventral and medial portion of the caudate nucleus (Ikemoto and Bonci, 2014; Sharma et al, 2018), and receives further innervation from multiple subregions, including the cortical regions which are important in processing sensory information, alongside the amygdala, a region involved in fear and sensory processing. The ventral striatum has been specifically shown to be involved in analysing the value of a potential reward and is reported to mediate the expectation of reward, and not the reward itself, which is supported by

previous studies using positron emission tomography (PET), which found DA release in the ventral striatum of patients with Parkinson's disease did not differ in those who experienced feelings of reward, and those who did report reward (Futente-Fernandez et al, 2002). This may justify why c-fos expression was not observed within this region following mephedrone alone, as acute treatment would not allow for the expectation of reward. However, the addition of caffeine produced a significant increase in c-fos expression in the ventral striatum, and due to the known involvement of the ventral striatum in reinforcement of additive stimuli, the increase in c-fos expression following this combination treatment suggests that caffeine exacerbates the positive reinforcement by increasing feeling of reward induced by mephedrone, and thus caffeine may increase the addiction potential to mephedrone and other psychostimulants. Specifically, it is known that DA release in the ventral striatum and Nac are vital for the rewarding reinforcement of psychostimulants (Veeneman et al, 2012; Biox et al, 1995; Burns et al, 1993). Therefore, the c-fos expression seen in the current study in response to combination treatment, but not either alone, is likely due to caffeine exacerbating mephedrone induced DA release in these regions. Caffeine administered alone has been shown to increase DA release in the VS/NacS (Solinas et al, 2002), and is therefore no surprise that it further elevates DA release, thus increases the rewarding properties compared to mephedrone alone. This is supported by previous studies who have found that caffeine exacerbates cocaine induced DA in the NacS (Galvalisi et al, 2017), and Vanattou-Saifoudine, Gossen and Harkin (2010) reported that caffeine increased MDMA induced DA release in the striatum. However, this study used *in vitro* brain tissue slices, which would therefore not contain the complete neuronal connections thus may not show the same effects that would display *in vitro*, alongside alterations in the structure due to the technical procedure (Dailet et al, 2002) so a microdialysis study performed *in vitro* would therefore be preferred. Furthermore, previous studies have reported that caffeine has been shown to

increase self-administration of cocaine in mice (Kuzmin et al, 2000) rats (Larson et al, 2018), and rhesus monkeys (Comer and Carroll, 1995). These results further highlight the increase in reinforcement caffeine administration has on psychostimulants.

The increase in c-fos expression following combined treatment in the dorsal striatum also has further implications for addiction. Alterations in the dorsal striatum have been linked to changes in habit learning, stimulus response and compulsive behaviours (Vollstädt-Klein et al, 2010), and damage to this region has been shown to disrupt cigarette smoking in those addicted (Jing et al, 2021), further highlighting the role the dorsal striatum plays in addiction. The increase in c-fos expression in this region suggests that mephedrone taken in conjunction with caffeine may exacerbate these effects, leading to an increase in the potential of users to become addicted to mephedrone, and other psychoactive substances that may be combined with caffeine.

4.2.1 Correlations observed in the dorsal striatum

C-fos expression in the dorsal striatum also displayed a significant correlation with hyperactivity, a result which has also been reported by Szucs et al, (2005), who also reported c-fos expression within this region was attenuated via 5-HT_{2A} antagonism, suggesting the serotonergic system, particularly the 5-HT_{2A} receptor, mediated c-fos expression within this region. Furthermore, pre-treatment with selective 5-HT_{2A} antagonists, in which these receptors are present in high concentrations throughout the striatum (Hanks and Gonzalez-Maeso, 2016) attenuate MDMA induced hyperactivity, further giving support to the 5-HT_{2A} system within the striatum mediating drug induced hyperactivity. However, Baumann, Clark and Rothman (2008) reported that MDMA produced a dose-dependent increase of DA within

the striatum, which was positively correlated with locomotor activity, which suggest that the DA system within the dorsal striatum may also be an important factor for drug induced locomotion. These results support the current study that the dorsal striatum is involved in both mephedrone induced stereotyped behaviour and locomotion, however further research needs to be completed to find out the specific role of 5-HT and DA in these behaviours. Alongside locomotion, c-fos expression in the dorsal striatum also displayed a correlation with head weaving. Previous studies have documented the role of the dorsal striatum in stereotypy, as lesions to the dorsal striatum by 6-OHDA decreased amphetamine induced stereotyped behaviour, however this effect was not seen by lesions with ibotenic acid to the same region (Antoniou et al, 1998), suggesting the dopaminergic system within the dorsal striatum is an important mediator for psychostimulant induced stereotypy.

4.3 Hypothalamus

In the current study, no treatment or treatment combinations induced statistically significant changes within the hypothalamus. The hypothalamus mediates many important physiological functions, including thermoregulation, hunger, sleep, thirst, and stress. In response to the latter, the hypothalamus regulates release of many hormones regulating the stress response (Heiman et al, 1997). As stress has been found to induce or trigger relapse in those with addictions (Covington and Miczek, 2001), it is quite surprising there was no significant c-fos expression, or correlations found in the current study, as it would be hypothesised mephedrone would affect this region. Furthermore, other similar studies have reported significant c-fos expression within the hypothalamus, further contradicting these results. For example, in response to mephedrone, Jones et al (2014) reported c-fos expression within the suprachiasmatic nucleus (SCN), but not other subregions of the hypothalamus. However, this

study used the Siberian hamster, and therefore species differences may account for contrasting results observed in the current study. Alongside being a key controller of the physiological processes mentioned above, studies have demonstrated the importance of the hypothalamus in reward seeking behaviours, due to its projections to key nuclei in the reward pathway, such as the prefrontal cortex, Nac and VTA (Yeoh et al, 2012). Furthermore, it is well documented that psychostimulants including cocaine (Lomax and Daniel, 1990), methamphetamine, (Behrouzvaziri et al, 2018) and MDMA (Green et al, 2014; Freedman, Johanson and Tancer, 2005) modulate thermoregulation, which is further influenced by ambient temperature (Dafters et al, 1995), consistent with the current finding of an increase in temperature in response to mephedrone due to the known modulation of body temperature by the hypothalamus. Previous studies have further documented the role the hypothalamus plays in response to drugs of abuse. For example, cocaine exposure induces plasticity via potentiating the excitatory drive within the hypothalamic neurons, specifically in the peripherical/lateral hypothalamus (Yeoh et al, 2012), so again, it was unexpected that mephedrone did not induce c-fos expression within this region.

4.4 Pre-frontal Cortex

As previously mentioned mephedrone alone did not induce significant c-fos expression in the mPFC. This was not hypothesised, as past literature has reported contrasting results, with c-fos expressed in the PFC following MDMA (Hunt et al, 2011; Marie-Claire et al, 2003) and methamphetamine (Thiriet, Zwiller Ali, 2001), amphetamine (Colussi-Mas et al, 2007) and cocaine (Marie-Claire et al, 2003). Furthermore, cocaine administration induced expression of the immediate early gene mkp1 within the PFC highlights the role of this region in drug induced effects and activation (Gao et al, 2017). Specifically, the current study observed c-fos

expression within the pre-limbic (PrL) region of the mPFC. Limpens et al (2014) identified the PrL as a key region involved in compulsive drug seeking, by pharmacologically inactivating the PrL with the use of GABA receptor agonists (baclofen and muscimol) which resulted in reduced suppression of cocaine and sucrose seeking in male Wistar rats, suggesting psychostimulants induce changes to this region, which may be important for the development of addiction. The PrL, alongside the infralimbic cortex, makes up the medial prefrontal cortex, which receives dopaminergic projections that arise within the VTA. Rats will self-administer drugs of abuse in the mPFC, such as cocaine (Goeders et al, 1985), implicating it in being an important region in mediating the rewarding effects of psychostimulants, which further contracting the results from the current study. The function of the PFC also makes the result from the current study more surprising, as PFC is a vital area for decision making based on potential reward (Bolton et al, 2018), which is expected, considering the PFC receives dense dopaminergic projections from the VTA, a vital area in the reward pathway. These projections predominantly innervate the infralimbic and prelimbic areas, and synapse onto pyramidal cells that project to the Nac (Carr et al, 1999). 80% of these VTA-Nac neurons are dopaminergic, with a combination of both D_1 and D_2 subtypes, (Boysibm McGonigle and Molinoff, 1986) highlighting the importance of the dopaminergic system in this pathway. The increased c-fos expression following combination treatment but not either drug alone in the mPFC has implications for psychostimulant addiction and adverse effects and provides further evidence towards the additive effect caffeine induces when combined with mephedrone. Caffeine (5 mg/kg) has been previously reported to increase cocaine (10mg/kg) induced c-fos expression in the PFC, in which significance was not observed with either drug alone (Muniz et al, 2017), which agrees with the results of the current study. Although much research is focused on the striatum in addiction, there is a large body of evidence (including the results from the current study) that the PFC is a key player in

mediating the effects induced by addictive drugs, and dysfunctions of this region can result in the debilitating symptoms seen in addicts. Although there is a low level of DA signalling within the PFC, it contains projections to a large range of regions, such as the amygdala, olfactory tubercle, the paraventricular, mediodorsal and reueins nucleus of the thalamus, and also key regions known to be involved in addiction responses, such as the VTA and NacC and NacS (Vertes, 2004), and thus is expected to be involved in the mediation of a wide range of responses. One process which has been attributed to the PFC is motivated behaviour, for example, Tran-Tru-Yen et al (2009) found using lesion techniques, that the prelimbic cortex plays a vital role in goal-directed responses. Therefore, mephedrone and other drugs of abuse that result in dysfunctions of the PFC may be responsible for drug seeking behaviours and motivation for taking the drug, even if this causes harm to the user and others around them, a key symptom of addiction. Furthermore, human imaging studies have implicated the involvement of this region in addiction, as neuroimaging studies have found PFC activation in response to cocaine craving and withdrawal (Goldstein and Volkow, 2011).

4.4.1 Correlations within the PFC

The magnitude of locomotion, stereotyped behaviour, and temperature change all correlated with c-fos expression in the mPFC, suggesting this region has an important role in the mediation of these responses. Although these behavioural and physiological responses are not directly associated with PFC function, due to the wide innovation of PFC projections to regions known for mediating these responses, the PFC likely has some indirect control over these effects. Previous studies have documented the tonic control of the PFC on the thermoregulatory neurons within the hypothalamus (Shibata et al, 1984), alongside containing specific neurons that respond to temperature changes throughout the body

(Shibata et al, 1988). The mPFC is a region that may regulate the mesolimbic dopamine system, which is known to play a vital role in stimulant induced locomotion (Hall, Powers and Gulley (2009), and so it is unsurprising the PrL displayed a positive correlation with drug induced locomotion. Psychostimulants are known to increase DA within the mPFC, in a dose-dependent manner (Moghaddam and Bunney, 1989), and intra-mPFC administration of quinpirole (5 nmol/side), a D_2 receptor agonist, attenuated cocaine (15mg/kg i.p.) induced locomotion (Beyer and Steketee, 2001). This effect was blocked by the D_2 antagonist sulpiride (1.5nmol/side), suggesting the D_2 receptors in the mPFC are responsible, at least in part, for the locomotor stimulatory effects induced by cocaine. Similarly, blockade of the D_1 receptors in the prelimbic region inhibits amphetamine (1.5mg/kg) induced locomotion (Mathews and McCormick, 2011). Alongside the DA system, the serotonergic system has also been implicated in drug induced locomotion, as a 90% depletion in 5-HT in the mPFC attenuated cocaine induced hyperactivity (Pum et al, 2008).

The correlation analysis also suggests the mPFC is also important in the stereotyped behaviour seen in the current study (i.e., head weaving). This was expected, as previous studies have documented the role the mPFC plays in the stereotyped responses to psychostimulant administration, which include head weaving and rearing in rats. Specifically, lesions to the PrL result in an increase in rearing (Tzschetke and Schmidt, 1998). The serotonergic system has been found to be vital in stereotyped behaviours induced by drugs, and correlates to the MDMA induced dose-dependent increase in 5-HT within the PFC (Baumann, Clark and Rothman, 2008). Furthermore, injection of phencyclidine into the PFC produces a higher level of head weaving when compared with an injection into the Cp or lateral ventricle, an effect which is attenuated with pre-treatment of the 5-HT synthesis inhibitor, p-chlorophenyl alanine (300 mg/kg) (Yamaguchi, Nabeshima and Kaemeyama,

1986), giving further support to the notion that the serotonergic system within the PFC is vital for mediating the stereotyped responses induced by psychostimulant administration. As mephedrone acts on SERT, inducing 5-HT release, it is reasonable to assume that this transmission in the PFC may account for the serotyped behaviour observed in the current study. Furthermore, caffeine has been shown to increase MDMA (20-50mg/kg) induced 5-HT release (Gorska and Golembiowka, 2015), and so the increase in head weaving in response to combination treatment is most likely the consequence of caffeine increasing 5-HT in the PFC. However, this study was performed in mice, and so similar studies should be repeated on rats using mephedrone administration confirm species comparability. This is important as distinct behaviours and metabolic rates have been noted between species as wel as strains within species. Further implicating the mPFC in stereotyped behaviour, lesions to the mPFC via quinolinic acid attenuate cocaine (10mg/kg) induced rearing (Tzschentke and Schmidt, 1998; Tzschentke and Schmidt, 2000). However, in contrast to the current study, Colussi-Mas and Schek (2008) also reported a significant c-fos expression, alongside correlations with locomotion, within the ventral pallidum, a subregion of the ventral striatum. Further contrasting with the results, the same study found a correlation between locomotion and c-fos expression within the hypothalamus, which was not observed in the current study. However, the difference in these results is most likely due to the pharmacological differences between MDMA and mephedrone, further highlighting the need to characterise the pharmacological profile of new and emerging drugs of abuse.

4.7 Overview

Mephedrone administration alone did not alter c-fos within the NacC, dorsal or ventral striatum, which is consistent with the idea that striatal subregions mediate different and

specific functions. However, c-fos expression might have been expected to increase throughout the striatum, due to its known involvement in addiction and results of previous studies (Quinn et al, 2018). For example, other groups showed mephedrone elevated c-fos expression has been observed in the ventral striatum, NacC, NacS and PFC (Jones et al, 2013; Motbey et al, 2011) unlike the current study with a more restricted pattern of c-fos expression. This disparity is most likely due to the higher doses used in these previous studies compared to those used in the current study, alongside the different routes of administration (15 and 30 mg/kg, i.p., compared to 10mg/kg, s.c.), so it is possible that more diverse c-fos expression would have occurred in the current study if a higher dose had been used. Furthermore, Jones et al (2013) used Siberian hamsters, and species differences therefore may influence the pattern of c-fos expression. Further contradicting the results, Cornish et al (2012) found methamphetamine (0.1mg/kg) induced c-fos expression in the caudate putamen (dorsal striatum) and NacC, but did not observe elevation in the NacS and the PFC, further highlighting the importance of profiling NPS, as they clearly display different patterns of activation.

We hypothesised that inhibition of the 5-HT_{1A} receptor by WAY-100635 would attenuate c-fos expression induced by mephedrone and caffeine co-administration, due to previous studies reporting that WAY-100635 influences mephedrone (Shortall et al, 2015), MDMA (Aguirre et al, 1998) and 8-OH-DPAT (Forster et al, 1995) induced hypothermia. However, WAY-100635 had no impact on the co-administration treatment induced c-fos expression, which due to the previous literature, was not hypothesised. 5-HT_{1A} agonists have been previously shown to decrease body temperature (Oostuska and Blessing, 2006), and WAY-100635 (0.1-0.4 mg kg-1, s.c.) has been previously shown to enhance hyperthermia induced by the 5-$HT_{2A/C}$ agonist DOI (0.025 1.6 mg kg-1, s.c.) (Salmi and Ahlenius, 1998), further

highlighting the importance of the 5-HT_{1a} receptor in thermoregulatory responses. This suggests that the 5-$HT_{2A/C}$ and the 5-HT_{1A} receptors interact in the modulation of body temperature, and perhaps combination treatment influences this interaction between these receptors, in which the 5-$HT_{2A/C}$ receptor primarily mediates this response, justifying why WAY-100635 had no impact on the thermoregulation.

Another justification as to why inhibition of the 5-HT_{1A} receptors by WAY-100635 did not produce the expected result (i.e., influence the behavioural and physiological effects induced by caffeine and mephedrone) is due to the mechanism of action of the antagonist at the 5-HT_{1A} receptors. WAY-100635 will bind to both post- and presynaptic receptors, and also auto receptors located in the dorsal raphe nuclei (Fletcher, Cliffe and Dourish, 1993; Henslet and Durgam, 2001), which ultimately acts to block further release of 5-HT. Therefore, inhibiting these auto receptors may act to increase extracellular serotonin, thus enhancing the effects induced by mephedrone and caffeine administration.

Research has documented the effect psychoactive drugs have on the serotonergic system, with cocaine administration results in an increase in 5-HT in the Nac (Parsons and Justice Jr, 1993; Andrews and Lucki, 2001; Reith et al, 1997; Teneud et al, 1996; as cited in Muller et al, 2007), as does amphetamine (Millan et al, 1999; Hernandez et al, 1987; Segal and Kcsenzki, 1997b); methamphetamine (Segal and Kuczenski, 1997b) and MDMA (Kankaanpaa et al, 1998), suggesting, the 5-HT system is important in mediating the effects induced by drugs of abuse, including mephedrone. However, it is believed that these effects, particularly the hypothermic response, is mediated by 5-HT transmission which is controlled by the 5-HT_{1A} auto receptors (Liang et al, 2017; Lin and Chuang, 2002) Furthermore, the

current literature suggests the 5-HT_{1A} receptor is important in mediating the effects induced by psychostimulants (Przegalinski, Siwanowicz and Filip,2006). However, the results clearly demonstrate that combination treatment of caffeine and mephedrone is not mediated by 5-HT_{1A} inhibition, as administration of WAY-100635 had no impact on c-fos expression. This suggests that the caffeine and mephedrone coadministration induced c-fos expression, are not mediated solely by the 5-HT_{1A} receptor, if at all.

Other receptors must be more important in mediating the effects of the combined treatment. Previous studies have documented the importance of the 5-HT_{1B} receptor in mediating responses elicited by mephedrone. For example, Shortall et al (2016) reported inhibition of the 5-HT_{1B} receptor (with the 5-HT_{1B} receptor antagonist (GR 127935; 3 mg/kg) attenuated mephedrone induced hyperactivity. Furthermore, 5-HT_{1B} knockout mice failed to induce a CPP following varying doses of cocaine (0-40mg/kg, i.p.) (Belzung, Scearce-Levie and Hen, 2000), and chronic, acute cocaine binging upregulated 5-HT_{1B} receptor by 80% in the NacC and DS (Hoplight, Vincow and Neumaier, 2007), further highlighting the importance of the 5-HT_{1B} receptor in the mediation of psychostimulant induced effects. Specifically, 5-HT_{1B} receptors in the Nac have proved particularly important, with activation of the 5-HT_{1B} receptors in the Nac reducing amphetamine self-stimulation (Fletcher, Azampanah and Korth, 2002), whilst also attenuating the response-potentiating effect of amphetamine (Fletcher and Korth, 1999). It is believed that 5-HT_{1B} located on GABAergic terminals that project from the Nac to VTA inhibit GABA release which ultimately disinhibit neurons within the VTA, inducing the effects mentioned above (Neumaier et al, 2002).

Furthermore, these effects may be attributed to mephedrone acting on the 5-$HT_{2A/2C}$ receptors, as mephedrone displays a high affinity for these receptors. Antagonism of the 5-HT_{2A} receptor by M100,907 (0.5mg/kg, s.c.) attenuated cocaine induced cocaine induced locomotion, whereas the 5-HT_{2C} antagonist SB242,084 (0.5 mg/kg i.p.) produced the opposing effect, potentiating cocaine induced locomotion (Fletcher, Grottick and Higgins, 2002).

4.6 Limitations of the study

Firstly, due to technical difficulties with the HPLC-ED, despite extensive work to fix the issues, it was impossible to analyse the *in vivo* microdialysis samples, and concomitantly determine levels of monoamine release from the striatum, which would have allowed further insight to the neurochemical effects of mephedrone and caffeine co administration compared with each treatment alone.

Had time permitted, the current MRes project could have undertaken further co-localisation immunohistochemical studies to identify the phenotype of neurons (i.e, serotonergic, glutaminergic or GABAergic) expressing c-fos, allowing a more in-depth insight into the neurons mediating the effects of mephedrone and caffeine combination to be identified.

Another limitation which is important to discuss is the use of only male rats. Male rats were used in the current study to avoid any sex differences (for example, hormonal effects) in the response to drug administration. However, other studies have reported that males and females respond differently to drug administration (Kennedy et al, 2013), and so the results of the

current study may be sex dependent. Furthermore, only adult rats were used in the current study, while a high percentage of users are adolescent, the findings may therefore not be applicable to these younger users. This is important, as previous literature has reported a clear sex differences in the neurochemistry of adolescent brains compared to adults, including altered expression in DA receptors (Rothman et al, 2012), and different responses to psychostimulants (Chitre, Bagwell and Murnane, 2020).

A relatively small sample size was used to examine c-fos immunoreactivity in the hypothalamus. To get accurate counts of cells expressing c-fos, at least five sections (with an aim of six) from each rat were analysed, and a resultant average calculated. However, due to technical difficulties with the sections breaking, it was impossible to get the desired number of hypothalamic sections from every rat, some only having one sections, making it impossible to average. Due to these difficulties, the hypothalamic data may not be representative, which may explain the unexpected results in the hypothalamus (see section 4.3). Furthermore, the sample size was determined to obtain significant behavioural results, rather than powered explicitly for differences in c-fos expression within brain regions. Therefore, a more powered study may be able to further identify differences between c-fos activation in these regions. In addition, the power may be confounded by using sub-optimal doses for the activation of psychostimulant brain circuits. As previously mentioned, (see section 4.1) previous research has determined only dosages of 75 mg/kg and up induce a significant change in c-fos expression (Bennett and Semba, 1998). Further work should be completed using higher dosages to examine the effect of caffeine on these brain regions.

Finally, c-Fos is a valuable tool to examine neuronal excitation, as basal expression is low it allows activated neurons to be mapped. However, it does not permit detection of neurons inhibited, and so neurons which are inhibited following mephedrone, caffeine or combination treatment which could occur in downstream pathways or following WAY-100635 administration. Finally, not all activated neuron express c-fos, and so it may not give a completely accurate picture of the neuronal pathways activated by mephedrone.

4.7 Relevance of the study

The results of the current study show that caffeine co-administration can exacerbate mephedrone induced c-fos expression, alongside hyperactivity and the conversion of hypothermia to hyperthermia, and induction of behaviours typical of serotonergic syndrome, which highlights the importance of considering the effects of caffeine as an adulterant in mephedrone, and abused psychostimulants, especially NPS. Furthermore, dysfunctions in these identified regions may provide evidence towards those predisposed towards drug addiction, which will ultimately act to reduce potential addiction, thus the adverse effects that follow, in human users.

4.8 Future studies

The current study clearly shows the potential adverse effects induced by co-administration and are clearly not mediated solely by the 5-HT_{1A} receptor, if at all. Therefore, future work should investigate downstream involvement of other monoamine receptors, including the dopaminergic receptor family, alongside other members of the serotonergic receptor family. Specifically, the 5-$HT_{2A/C}$ receptor appears to be important in mediating the effects of

psychostimulants (see section 4.4), thus should be researched in further detail. Alongside this, the effects of mephedrone and caffeine should be studied on females and differing age groups to increase generalizability to the entire population, as previous research has demonstrated a clear sex (Castro-Zavala, Sanchez and Valverde, 2020; Jackson, Robinson and Becker, 2006; Marsh et al, 2018) and age difference in the response to psychostimulants (Yang et al, 2011; McDougall et al, 2014).

Finally, the neurotoxic effects of mephedrone, and the combination of mephedrone and caffeine, alongside the long-term effects were not observed in the present study. Previous research in both rodents and humans have highlighted the long term effects induced by psychostimulants (Creighton, Black and Hyde, 2018; Bolla, McCann and Riacurte, 1998;Bowyer et al, 1998), and it is therefore vital that more research is conducted to examine the long term effects induced by the combination of caffeine and mephedrone.

4.9 Conclusions

The current study has highlighted that the additive effects of mephedrone and caffeine, parallel findings with MDMA and caffeine (Vanattou-Saifoudine et al, 2012). These results suggest a potential for additional adverse effects in recreational users, which highlights the importance of understanding the pharmacology of co-administered drugs of abuse, to analyse their potential interaction and adverse effects, to help development of effective treatments for the adverse effects of psychostimulants, with the goal to reduce harm in human users. This is especially important in recreational drugs of abuse which are often combined with adulterants, like caffeine, although this study did not identify the receptors involved in the downstream effects induced by combined treatment, as unlike mephedrone alone (Shortall et

al, 2015) these appear insensitive to 5-HT_{1A} receptor blockade, it will permit future studies to identify the specific receptors involved, by inhibiting other potential targets.

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