Caffeine alters the behavioural and body temperature responses to mephedrone without causing long-term neurotoxicity in rats

Short title: Caffeine and mephedrone co-administration

SE Shortall, AR Green, KCF Fone, MV King*

School of Life Sciences, Medical School, Queen's Medical Centre, The University of Nottingham, Nottingham, NG7 2UH

*Corresponding author. Tel.: +44 1158 230154; fax: +44 1158 230142

Email: madeleine.king@nottingham.ac.uk (M.V. King)

Abstract

Administration of caffeine with 3,4-methylenedioxymethamphetamine (MDMA) alters the pharmacological properties of MDMA in rats. The current study examined whether caffeine alters the behavioural and neurochemical effects of mephedrone, which has similar psychoactive effects to MDMA. Rats received either i.p. saline, mephedrone (10mg/kg), caffeine (10mg/kg) or combined caffeine and mephedrone twice weekly on consecutive days for three weeks. Locomotor activity (days 1 and 16), novel object discrimination (NOD, day two), elevated plus maze (EPM, day eight) exploration, rectal temperature changes (day nine) and prepulse inhibition of acoustic startle (PPI, day 15) response were assessed. Seven days after the final injection, brain regions were collected for measurement of 5-hydroxytryptamine (5-HT), dopamine and their metabolites. Combined caffeine and mephedrone further enhanced the locomotor response observed following either drug administered alone, and converted mephedrone-induced hypothermia to hyperthermia. Co-administration also abolished mephedrone-induced anxiogenic response on the EPM but had no effect on NOD or PPI. Importantly, no long-term neurotoxicity was detected following repeated mephedrone alone or when co-administered with caffeine. In conclusion, the study suggests a potentially dangerous effect of concomitant caffeine and mephedrone, and highlights the importance of taking polydrug use into consideration when investigating the acute adverse effect profile of popular recreational drugs.

Keywords: Mephedrone, caffeine, locomotor activity, body temperature, anxiety

Introduction

There has been an alarming increase in the appearance of novel psychoactive substances (NPS) in the last few years, many based on amphetamine- and cathinone-type chemical structures and therefore presumably produced and ingested in the expectation that they will emulate the psychoactive properties of established recreational drugs such as methamphetamine and 3,4-dimethoxymethamphetamine (MDMA or ecstasy). There is little or no pharmacological or toxicological knowledge of most of these NPS, and predictions cannot be made solely on the basis of information on related and more established drugs (Dal Cason et al., 1997; Shortall et al., 2013a; Glennon, 2014). A further complication is that few users ingest only the drug under investigation, since almost all recreational drug users ingest other psychoactive compounds either knowingly (Curran, 2000; Cole and Sumnall, 2003) or because of adulteration of a "named" drug by the addition of other pharmacologically active substances (Vogels et al., 2009). Consequently while preclinical studies may indicate that the NPS under investigation is unlikely to produce severe acute or long-term toxicological problems when ingested at modest dose, this may not reflect the situation when the drug is taken by a user who has also consumed other psychoactive or even non-psychoactive substances (Green and Nutt, 2014).

One non-psychoactive substance that is ubiquitous, but widely ignored in preclinical evaluation, is caffeine. Its presence not only in coffee and tea, but also in cola, and particularly in the newer 'energy' drinks such as Red Bull and many other brands with high caffeine content, means that it is often ingested with NPS. The effects of high caffeine ingestion and the problems of combining caffeine with alcohol have been detailed by Reissig et al. (2009). More recently Vanattou-Saïfoudine et al. (2012) have reviewed evidence that in rats caffeine can increase the toxicity of a variety of psychostimulants including 3,4-

methylenedioxymethamphetamine (MDMA, ecstasy), typically enhancing hyperthermia, tachycardia and seizures and also long term serotonergic neurotoxicity.

A popular NPS that has received much interest over the last 6 years is the synthetic cathinone derivative, 4-methylmethcathinone (mephedrone), which was first introduced as a 'legal high' and received substantial media attention due to its possible involvement in a number of adverse events in users, thereby leading to its reclassification as a controlled substance in many countries (Dargan et al., 2011; Gershman and Fass, 2012). Despite being banned, mephedrone remains available for illicit recreational consumption (Ayres and Bond, 2012; Van Hout and Brennan, 2012; Yamamoto et al., 2013; Salomone et al., 2015) and many users have compared its psychostimulant effects to those of MDMA and cocaine (Kelly et al., 2013; Green et al., 2014). Since caffeine produces marked enhancement of the adverse effects of MDMA we have now examined whether it also alters the pharmacological changes associated with mephedrone administration.

Caffeine primarily elicits its psychostimulant effects via modulation of dopamine transmission by antagonism of the A_1 and A_{2A} adenosine receptors, as well as by phosphodiesterase inhibition (for review see Fisone et al. 2004). Adenosine A_1 receptors have a widespread distribution in the brain, with highest levels in the hippocampus, cerebral cortex, hypothalamus and cerebellum, and low levels in the basal ganglia, while A_{2A} receptors are found mainly in dopamine rich brain regions such as the striatum and nucleus accumbens. It is the effect of caffeine on dopamine release that is commonly attributed to the toxic effects (hyperthermia, cardiovascular toxicity and seizure threshold) of its co-administration with MDMA (Vanattou-Saifoudine et al., 2010b; Vanattou-Saifoudine et al., 2010a).

Mephedrone is structurally similar to MDMA and both compounds have a high affinity for the dopamine, 5-HT and noradrenaline uptake transporters and systemic administration to

rats elevates extracellular monoamine levels (Kehr et al., 2011; Baumann et al., 2012). While it is well established that MDMA causes neurotoxic loss of 5-HT in the rat, several studies have failed to find similar neurotoxicity following repeated mephedrone administration (for review see Green et al., 2014). Mephedrone causes hyperactivity and hypothermia, hippocampal-dependent contextual association and short-term memory deficits in rats (Kehr et al., 2011; Baumann et al., 2012; Lisek et al., 2012; Wright et al., 2012; Miller et al., 2013; Shortall et al., 2013a; Shortall et al., 2013b; Golembiowska et al., 2015; Shortall et al., 2015), while human users report cold/blue fingers as well as hot flushes and sweating (indicative of changes in peripheral vascular tone which controls thermoregulation), anxiety, impaired short-term memory and poor concentration (Schifano et al., 2011; Freeman et al., 2012). It is important to investigate any potential adverse effects of mephedrone and caffeine co-administration in the rat since the co-administration of caffeine with MDMA exacerbates the effects of MDMA on monoamine release (Gorska and Golembiowska, 2015), increased dopamine is related to the additive toxic effects of caffeine with MDMA, and previous studies have shown that dopamine also plays an important role in mephedrone-induced changes in body temperature and locomotor activity (Lisek et al., 2012; Shortall et al., 2013a; Shortall et al., 2015). Therefore the current study examined the effects of combined caffeine and mephedrone on locomotor activity (LMA), novel object discrimination (NOD), elevated plus maze (EPM) exploration, rectal temperature and prepulse inhibition of acoustic startle (PPI) response.

In the current study, rats received twice weekly injections of caffeine and mephedrone, alone or in combination, on consecutive days a week to mimic the typical recreational weekend dosing employed by human users. The selected mephedrone dose produces sub-maximal changes in rectal temperature and locomotor activity (Green et al., 2014) and importantly appears to have translational relevance to human recreational doses (Shortall et

al., 2013a; Shortall et al., 2013b; Green et al., 2014). The caffeine dose was also selected from previous reports examining its interaction with other psychoactive drugs (Vanattou-Saifoudine et al., 2012).

Methods

Animals

Adult male Lister hooded rats (210-250g; Charles River UK) were housed in groups of four in wire top cages in Scantainer ventilated cabinets under constant environmental conditions (12 h light/dark cycle with lights on at 07.00 h, ambient temperature 21 ± 2 ˚C and relative humidity 55 ± 10%), with food and water freely available. All experiments were performed during the light phase between 09.00 h and 16.00 h. All procedures were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and ARRIVE guidelines, with approval of the University of Nottingham Local Ethical Committee. The injections and dose schedule were chosen to comply with the three R's of humane animal testing. All measurements were performed by an observer who was unaware of the treatments administered.

Drugs and experimental protocol

Rats (n=8 per treatment group) received saline vehicle (1 ml/kg i.p.), (±)-mephedrone HCl (10 mg/kg i.p., Ascent Scientific), caffeine (10 mg/kg i.p., Sigma Aldrich) or a combination of caffeine and mephedrone in the same solution (both 10 mg/kg) at these doses twice weekly on consecutive days for three weeks to mimic weekend recreational use in humans. On each stated dosing day rats were assessed for LMA (days one and 16), NOD (day two), EPM behaviour (day eight), rectal temperature changes (day nine) and PPI response (day 15). These behavioural tasks were selected because of their translational relevance to the drug

effects reported by human users and were measured in order of least to most aversive to minimise exposure to the previous paradigm on measurements obtained; an approach successfully used in one of our previous mephedrone and MDMA studies (Shortall et al., 2013b). All apparatus was cleaned with 20% ethanol between tests to remove any odour cues.

Seven days after the final injection, brain regions were collected for measurement of monoamine neurotransmitters and their major metabolites by high performance liquid chromatography with electrochemical detection (HPLC-ED) to determine any potential neurotoxicity that may have occurred.

Locomotor activity

LMA was measured following the first injection (day one) to assess drug-induced changes in motor activity and reassessed following the sixth injection to identify any drug-induced sensitisation or tolerance that may have developed with additional injections (Shortall et al., 2013b). Rats were placed into an individual perspex arena and allowed to habituate for 60 min then quickly injected and returned into the same arena for a further 60 min. LMA was recorded pre- and post-injection using a photobeam activity system (San Diego Instruments) consisting of eight parallel beams to record horizontal ambulations with a single ambulation count being defined as two consecutive beam breaks. Cumulative activity counts were recorded in 5 min epochs. Four upper parallel upper beams recorded rearing behaviour and a single rear count was generated from a single beam break.

Novel object discrimination

Visual working memory is impaired in intoxicated mephedrone users (Freeman et al., 2012) therefore NOD was assessed following the second injection (day two) to compare drug-induced changes in visual recognition memory using previously described methods (King et

al., 2004; Shortall et al., 2013b). In summary, rats were returned to the same arena as the previous day, 26 min post-injection, for an additional 3 min habituation. This was followed by 1 min in the home cage and two subsequent 3 min object exploration trials separated by a 2 h inter-trial interval. In the first (familiarisation) trial, exploration of two identical objects (plastic bottles covered in white masking tape) was recorded. For the second (choice) trial, one of these objects was replaced with a novel object of the same size and shape but with four additional horizontal stripes of black electrical insulation tape. Exploration of the objects was recorded using stopwatches and defined as sniffing, licking, chewing or having moving vibrissae whilst directing the nose towards and <1 cm from an object. Climbing on an object in the absence of directed interest was not recorded as exploration. Actual times spent exploring the objects in the choice trial were used to calculate the discrimination ratio (novel/[novel + familiar time]) for further analysis and a ratio >0.5 was used to define successful discrimination.

Elevated plus maze

Since mephedrone is anxiogenic in human users (Schifano et al., 2011), EPM exploration was measured following the third injection (day eight) using an established protocol (Bull et al., 2004) to examine any potential drug-induced 'anxiety-related' behaviour. The black Perspex maze was elevated 70 cm above the floor and consisted of four arms (45 cm) arranged at right angles around a central square (10 cm x 10 cm). Light intensity on the two closed arms (30 cm walls) was 20 lux and on the two open arms (no walls) was 60 lux. Rats were placed onto the centre of the EPM facing a closed arm 30 min post-injection. Exploration was recorded for 5 min using Ethovision XT 7 software (Noldus, UK). Measures recorded were total time spent in open and closed arms, time and percentage time spent in open arms ([time spent in open arms/total time spent in arms] x 100). Frequency of unprotected head dips

(where the whole head was lowered beneath the edge of the open arm) and stretch attend postures (movement of both forepaws into an open arm without simultaneous movement of the hindpaws) were scored manually using the computer keypad by an observer in a separate room.

Rectal temperature

Mephedrone causes hypothermia in rats (for review see Green et al., 2004) and human users report changes in thermoregulation (Schifano et al. 2011). Therefore, drug-induced changes in rectal temperature were recorded following the fourth injection (day nine) as previously described (Shortall et al., 2013a). The experimental room temperature was 19.6 ± 0.3°C with rats present in the same arena as used for LMA and NOD. The initial rectal temperature was recorded 40 min prior to injection (rectal probe, Portec Instrumentation, Bedfordshire, UK) to familiarise rats to the procedure. Baseline temperatures were recorded at the time of injection and then at 20 min intervals for 2 h post-injection. The probe was lubricated with sterile paraffin and inserted approximately 6 cm into the rectum and allowed to stabilise for approximately 20 s before each reading.

Prepulse inhibition of acoustic startle response

PPI is a measurement of sensorimotor gating (the brain's ability to modulate its sensitivity to incoming sensory stimuli) which is known to be impaired in rats following MDMA administration (Vollenweider et al., 1999), therefore PPI was measured following the fifth injection (day 15) to assess drug-induced changes in sensorimotor gating, using an established protocol (Jones et al., 2011; Shortall et al., 2013b). Test sessions commenced 30 min after injection and consisted of 5 min acclimatisation to background white noise (62 dB), followed by ten successive startle-alone pulses (120 dB) and a further 50 startle trials (ten

without any pre-pulse and 40 preceded by 72, 76, 80 and 84 dB pre-pulses in a pseudorandom order and with an unpredictable inter-trial interval) and ending with five startle-alone pulses. The 72 dB pre-pulse was used as a primer but resultant data were not included in analysis. Individual whole body startle responses were recorded for 100 ms from the initiation of the startle pulse using Startle Reflex Testing software (San Diego Instruments, CA) to provide a total cumulative area under the curve (AUC) response for each trial. These data were used to calculate percentage PPI for each pre-pulse intensity (after applying a conditional statement to eliminate any extreme values greater than ± 2 standard deviations from the mean which might result from non-startle related movement of the rat within the tube) using the equation percentage PPI = ((pulse alone AUC - pre-pulse AUC)/pulse alone AUC) x 100.

Measurement of brain monoamines

Rats were killed by concussion and immediate decapitation seven days after the last of six injections to measure any long-term neurotoxic changes in brain monoamine levels. The hypothalamus and right striatum, frontal cortex and hippocampus were rapidly dissected on a refrigerated table (4˚C, Osborne Refrigeration, Sussex, UK), weighed, flash frozen in liquid nitrogen and stored at -80˚C until analysis. HPLC-ED was performed as previously described (Shortall et al., 2013a; Shortall et al., 2013b). Tissue samples were sonicated (Soniprep 150: MSE Scientific instruments, UK) in 800 µl of 0.1 M perchloric acid containing 0.4% w/v sodium metabisulfite, centrifuged at 17400 x g, 4 ˚C for 20 min and the supernatant filtered through a 0.45 µm syringe tip filter (Kinesis Ltd, UK). Five µl of sample was injected into the Targa C18 3 µm column (10 cm x 2.1 mm; Phenomenex) and dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were detected at a potential of +0.59 V using an electrochemical detector (Antec-Leyden, The Netherlands). Mobile phase consisting of 50

mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 8 mM potassium chloride, 0.15 mM octanesulfonic acid and 10% methanol (pH 3.8-4) was circulated (Dionex Ultimate 3000) through the system at a flow rate of 0.2 ml/min. Quantification was performed using Galaxie chromatography workstation against standards.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (v6.02) and SPSS (v21) software. LMA, NOD, rectal temperature and PPI data were analysed using three-way repeated analysis of variance (ANOVA) with caffeine and mephedrone treatment as between-group factors and time (LMA, temperature), object (NOD) or pre-pulse amplitude (PPI) as the within-group factor. NOD choice trial discrimination ratio, EPM and HPLC data were analysed by two-way ANOVA with caffeine and mephedrone treatment as between-group factors. In each case, Bonferroni multiple comparison post-hoc tests were used and $p<0.05$ was considered statistically significant. All data are presented as mean ± SEM.

Results

Locomotor activity

As expected all rats showed a progressive decline in locomotor activity over the first 60 min on both LMA test days (data not shown) confirming habituation to the arena prior to drug administration. On day one of testing both mephedrone and caffeine elevated total locomotor activity over the 60 min following injection above vehicle control levels (Figure 1). Combined administration of caffeine with mephedrone also prolonged the duration of mephedrone-induced hyperactivity, such that the combined group displayed greater activity than mephedrone alone at 60 min post-injection and vehicle from 10-60 min post-injection (mephedrone x caffeine x time interaction ($F(11,308)=4.16$, $p<0.001$; Figure 2a), without

enhancing the magnitude of the peak locomotor activity response observed following mephedrone alone.

Following the sixth injection (day 16) both mephedrone and caffeine again enhanced the total locomotion over 60 min (Figure 1) and co-administration of caffeine and mephedrone again caused a marked increase in activity over the duration of testing (Figure 1) which was significantly greater than vehicle from 5-60 min and from mephedrone alone at 60 min post-injection ($F(11,308)=4.39$, $P<0.001$; Figure 2b). There was no significant difference in the total horizontal locomotion between the first and sixth injections for any treatment group (mephedrone x caffeine x day interaction: $F(1,28)=0.11$, $p>0.05$).

There was no significant effect of any drug treatment on rearing behaviour on either test day ($P>0.05$; data not shown) nor was there any difference in the total number of rears between the first and second locomotor test days ($P>0.05$; day one and day 16).

Novel object discrimination

NOD was assessed following the second injection (day two). There was no spatial preference for either object during the familiarisation trial for any treatment group (mephedrone x caffeine x object $F(1,28)=0.04$, $p>0.05$), however, mephedrone treated rats spent significantly less cumulative time exploring both objects than vehicle treated rats ($F(1,28)=9.05$, $p<0.01$, data not shown). Vehicle treated rats successfully discriminated the novel from the familiar object during the choice trial such that there was a significant main effect of object ($F(1,28)=4.81$, $p<0.05$), while mephedrone, caffeine and combined mephedrone and caffeine treated rats failed to discriminate the two objects (mephedrone x caffeine x object: $F(1,28)=0.47$, $p>0.05$, Figure 3a). However, there was no significant effect of mephedrone, caffeine or their combination on the discrimination ratio (mephedrone x caffeine: $F(1,28)=0.47$, $p>0.05$, Figure 3b).

Elevated plus maze

On day eight following the third drug administration, co-administration of caffeine and mephedrone increased time spent on the open arms compared to mephedrone alone (mephedrone x caffeine interaction: $F(1,28)=4.62$, $p<0.05$, Figure 4a) without altering the percentage number of entries into the open arms of the EPM (mephedrone x caffeine interaction: ($F(1,28)=0.44$, $p>0.05$). Concomitant administration of mephedrone and caffeine also prevented the mephedrone-induced decrease in exploratory head dips, while increasing the number of stretch attend postures compared to vehicle control, in a similar manner to mephedrone alone ($p<0.05$, Figure 4b, 4c). Caffeine alone did not alter the number of stretch attend postures ($F(1,28)=1.02$, $p<0.05$) or head dips ($F(1,28)=1.02$, $p<0.05$). Examination of the ethological behaviours on the EPM supported the observation from exploratory time that there was a switch from anxiogenic to anxiolytic profile when mephedrone was administered with caffeine.

Rectal temperature

There was no significant difference in baseline temperature immediately prior to the fourth injection on day nine (T=0 min; vehicle: 39.1±0.2°C, mephedrone: 39.3±0.1°C, caffeine: 39.1±0.1°C, mephedrone+caffeine: 39.1±0.1°C, $p>0.05$, mean ± SEM). Mephedrone decreased rectal temperature from 20-60 min post-injection but in marked contrast co-administration of mephedrone with caffeine increased rectal temperature significantly above that following either mephedrone alone (from 60-120 min post-injection) or vehicle (120 min post-injection; mephedrone x caffeine x time interaction: $F(6,168)=2.38$, $p<0.05$, Figure 5). Thus the hypothermia produced by mephedrone was converted to hyperthermia when co-administered with caffeine (which itself did not alter temperature).

Prepulse inhibition to acoustic startle

On day 15, following the fifth injection, all treatment groups exhibited the normal attenuation of startle by exposure to increasing pre-pulse amplitude (pre-pulse: $F(2,48)=60.86$, $P<0.001$, data not shown). There was no difference in basal reactivity or habituation to the startle pulse alone. Neither mephedrone nor caffeine alone or in combination had any effect on percent PPI (mephedrone x caffeine x pre-pulse interaction: $F(2,48)=0.09$, $p>0.05$, Figure 6).

Neurochemistry

There was no significant effect of mephedrone alone, or in combination with caffeine, on *ex vivo* levels of dopamine, 5-HT or their major metabolites (3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA)) in the hypothalamus, striatum, frontal cortex or hippocampus collected seven days after the last of six injections. The only significant change was that caffeine alone increased dopamine levels (58.8 ± 4.3 pmol/mg, mean ± SEM, Table 1) in the striatum compared to vehicle controls (34.5 ± 5.9 pmol/mg; $F(1,28)=5.38$, $p<0.05$).

Discussion

The main findings of this study were that caffeine co-administration had an additive effect on mephedrone-induced hyperactivity, and converted mephedrone-induced hypothermia to hyperthermia. It also attenuated ‘anxiety-like’ behaviours on the EPM. No evidence was found to suggest that the combination induced neurotoxicity in either 5-HT or dopamine neuronal systems the brain. These findings are now discussed in more detail.

The increase in locomotor activity by amphetamine derivatives is generally accepted to be due to drug-induced release of dopamine in the nucleus accumbens (Anden and Jackson,

1975; Parkinson et al., 1999; Boye et al., 2001). It seems reasonable to suggest the same mechanism is also involved when mephedrone is administered to rats, because this drug also enhances dopamine release in that region (Kehr et al., 2011). This conclusion is not weakened by our recent work which observed that intraventricular 6-hydroxydopamine (6-OHDA) administration failed to attenuate the increased locomotion induced by mephedrone (Shortall et al., 2015) because the n. accumbens is resistant to 6-OHDA-induced dopamine denervation (Meredith et al., 1995; Boye et al., 2001). There is also evidence that caffeine enhances dopamine release in this brain region and does so by blocking adenosine A_1 receptors (Solinas et al., 2002). The proposal that psychostimulants such as the amphetamines and caffeine are enhancing locomotor activity through the same mechanism in the same region of the brain (Solinas et al., 2002) therefore seems reasonable and although no enhancement of the peak locomotor activity was seen this may be because of a ceiling effect. We therefore suggest that mephedrone and caffeine are also acting through the same mechanism of dopamine release in the n. accumbens. This would likely result in the hyperlocomotor effect being additive rather than potentiated, which is indeed what we observed and also that there would be cross-tolerance between caffeine and psychostimulants which has also been observed previously (Mumford and Holtzman, 1991). The fact that mephedrone-induced hyperactivity is attenuated by 5-HT lesioning with 5,7-dihydroxytryptamine and 5-HT antagonists (Shortall et al., 2015) in no way weakens this proposal since enhanced locomotor activity following administration of amphetamine-like drugs is known to be modulated by changes in 5-HT function including inhibition by 5-HT_{1B} receptors (Bankson and Cunningham, 2002) as we also found with mephedrone (Shortall et al., 2015). Additionally, it is unlikely that caffeine is influencing the metabolism of mephedrone as caffeine and mephedrone are primarily metabolised by different cytochrome

P450 isozymes, CYP1A2 and CYP2D6, respectively (Kot and Daniel, 2007; Pedersen et al., 2013).

Evidence for repeated mephedrone administration producing sensitisation of the locomotor response to subsequent injections of the drug is contradictory. We have previously observed this phenomenon (Shortall et al., 2013b) as did Lisek et al. (2012) while Motbey et al. (2012) failed to observe sensitisation. Such conflicting results may relate to sensitisation being very dependent on both timing of administration (number of injections and intervals between injections and testing) and the doses employed. In the current study we again failed to observe sensitisation even though a similar protocol was used to our earlier study (Shortall et al., 2013b). What is nevertheless clear is that caffeine and mephedrone continued to produce a similar enhanced locomotor response to that of mephedrone alone even when both drugs had been given six times over the three week period.

MDMA can induce severe hyperthermia in rats particularly when they are housed in groups (see Green et al., 2003). When caffeine is administered together with MDMA it results in a further enhancement of the hyperthermic response which can prove fatal (Green et al., 2003; McNamara et al., 2006; Vanattou-Saifoudine et al., 2012). However, when MDMA is given to individually housed rats it generally results in hypothermia (Bull et al., 2004; Docherty and Green, 2010). Mephedrone administration also induces hypothermia even when the rats are grouped or exposed to a high ambient temperature (Green et al., 2014). The hypothermic response of individually housed rats given MDMA is switched to hyperthermia when caffeine is co-administered (McNamara et al., 2006) and similarly we now find that mephedrone-induced hypothermia is switched to hyperthermia when caffeine is also injected. There is substantial evidence that dopaminergic mechanisms are involved in MDMA-induced temperature changes, both hypothermia and hyperthermia (Docherty and Green, 2010). In contrast, evidence suggests that serotonergic mechanisms are key to the hypothermic effect of

mephedrone (Shortall et al., 2015). Nevertheless the fact that 5-HT is also involved in modulating the temperature changes induced by MDMA (Saadat et al., 2005) indicates that both neurotransmitters are likely to be involved in thermoregulation following the administration of both MDMA and mephedrone. Perturbing the balance between the magnitude of dopamine and 5-HT being released by mephedrone by further enhancing dopamine release by injection of caffeine is therefore likely to alter the temperature response observed when only mephedrone is injected. There are clinical reports of both hypothermia (blue fingers) and hyperthermia (sweating) in recreational mephedrone users (Winstock et al., 2011; Wood and Dargan, 2012) and one can speculate that these apparent contradictions might result from whether caffeine has or has not also been taken.

Caffeine administration also increased the neurotoxic loss of 5-HT in the forebrain produced by MDMA (McNamara et al., 2006). We previously failed to find evidence that mephedrone is neurotoxic at the doses employed in this study (Shortall et al., 2013b) and the current results have confirmed this. Caffeine co-administration with mephedrone did not result in neurotoxicity being observed seven days after the final administration.

Repeated doses of mephedrone do not result in a long-term change in 'anxiety-like' behaviour in rats or mice on an EPM ten or more days after the last dose (Motbey et al., 2012; den Hollander et al., 2013). However there do not appear to have been any studies on anxiety-like behaviour immediately following the drug as undertaken here. Results obtained in the EPM suggested a tendency towards an increase in anxiety-like behaviour following mephedrone injection; although mean time on the open arms was not significantly decreased there were significant decreases in head dips and increases in stretch attend postures, these two behaviours being thought to reflect exploratory open arm avoidance and risk assessment, respectively (Bailey and Crawley, 2009). Mephedrone therefore appears to have an acute anxiogenic profile as also seen following MDMA (Ho et al., 2004). Interestingly, when

combined with caffeine this anxiogenic profile was reversed such that rats spent more time on the aversive open arms and made more high risk head dips than rats treated with mephedrone alone. The number of stretch attend postures was unaffected by caffeine co-administration, suggesting combined treatment reduced exploratory open arm avoidance. Anxiety is a self-reported adverse effect of mephedrone administration in human users (Schifano et al., 2011) so translationally, if caffeine also prevents the anxiogenic effects of mephedrone in users then this may make caffeine and mephedrone co-administration more desirable to users.

In agreement with our earlier study mephedrone impaired NOD and failed to influence PPI (Shortall et al., 2013b). Caffeine had a similar profile and did not alter the mephedrone responses in these two tests.

In conclusion evidence suggests that co-administration of caffeine with mephedrone produces marked change in locomotor and body temperature response to mephedrone. The changes are generally consistent with the altered responses seen when caffeine is given with MDMA, and a recent study has also demonstrated the toxic effects of combined ethanol and mephedrone in the mouse (Ciudad-Roberts et al., 2015), further highlighting the importance of taking polydrug use into consideration when investigating the adverse effect profile of these commonly used recreational drugs as has been emphasised before (Green and Nutt, 2014).

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Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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Table 1. *Ex vivo* brain tissue dopamine and 5-HT levels.

Tissue levels (pmol/mg tissue)				
	Fr Crtx	Hippoc	Str	Hypo
	Dopamine			
V	0.93 ± 0.1	0.46 ± 0.1	34.50 ± 5.9	3.37 ± 0.7
Meph	0.98 ± 0.2	0.34 ± 0.1	51.72 ± 2.5	2.67 ± 0.2
Caff	0.95 ± 0.1	0.28 ± 0.1	**58.81 ± 4.3***	3.53 ± 0.9
Meph+Caff	0.90 ± 0.1	0.63 ± 0.3	46.91 ± 5.4	3.30 ± 0.6
	5-HT			
V	10.65 ± 1.1	0.30 ± 0.6	3.15 ± 0.2	8.02 ± 1.5
Meph	10.61 ± 1.0	3.43 ± 0.5	3.85 ± 0.3	6.04 ± 1.0
Caff	11.12 ± 0.5	3.00 ± 0.6	3.79 ± 0.3	9.36 ± 2.2
Meph+Caff	12.41 ± 0.4	3.41 ± 0.6	3.95 ± 0.3	8.61 ± 2.0

Neurotransmitter levels were measured 7 days after the last i.p. injection of saline vehicle (V; 1 ml/kg), mephedrone (Meph; 10 mg/kg), caffeine (Caff; 10 mg/kg) or combined mephedrone and caffeine (Meph+Caff; both 10 mg/kg) to adult male Lister hooded rats (n=8 per treatment group) which were injected on two consecutive days for each of three weeks (six injections total). Data are expressed as mean ± SEM. *$p<0.05$ compared to vehicle, Bonferroni multiple comparisons post-hoc following two-way ANOVA.

Figure 1. Total cumulative activity counts following acute and repeated intermittent i.p. injection of saline vehicle (V, 1 ml/kg), mephedrone (Meph, 10 mg/kg), caffeine (Caff, 10 mg/kg) or co-administration of mephedrone and caffeine (Meph+Caff, both 10mg/kg) twice weekly on consecutive days a week for three weeks to adult male Lister hooded rats (n=8 per treatment group). Locomotor activity was measured on day one (a) and day 16 (b) following the first and sixth challenge injections. Data are displayed as mean ± SEM. *p<0.05, ***p<0.001 compared to vehicle; †p<0.05, †††p<0.001 combined caffeine and mephedrone compared to caffeine alone; ‡p<0.05 combined mephedrone and caffeine compared to mephedrone alone, Bonferroni multiple comparisons post-hoc following three-way repeated measures ANOVA.

Figure 2. Locomotor effects of acute (a) and repeated intermittent (b) i.p. injection of saline vehicle (V, 1 ml/kg), mephedrone (Meph, 10 mg/kg), caffeine (Caff, 10 mg/kg) or co-administration of mephedrone and caffeine (Meph+Caff, both 10mg/kg) twice weekly on consecutive days a week for three weeks to adult male Lister hooded rats (n=8 per treatment group). Locomotor activity was measured on day one (a) and day 16 (b) following the first and sixth challenge injections. Data are displayed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle; †p<0.05, ††p<0.01, †††p<0.001 combined caffeine and mephedrone compared to caffeine alone; ‡p<0.05, ‡‡p<0.01 combined mephedrone and caffeine compared to mephedrone alone, Bonferroni multiple comparisons post-hoc following three-way repeated measures ANOVA. The bars indicate significance from vehicle for both mephedrone alone and in combination with caffeine.

Figure 3. Effect of caffeine on mephedrone-induced changes in object exploration during the familiarisation trial (a) and choice trial discrimination ratio (b) of the novel object

discrimination (NOD) task. Adult male Lister hooded rats (n=8) received i.p. saline vehicle (V, 1 ml/kg), mephedrone (Meph, 10 mg/kg), caffeine (Caff, 10 mg/kg) or combined mephedrone and caffeine (Meph+Caff, both 10 mg/kg) twice weekly on consecutive days for three weeks. NOD was measured on day two, following the second injection. Data are displayed as mean ± SEM. *p<0.05 compared to familiar object for that treatment group, Bonferroni multiple comparisons post-hoc following three-way repeated measures ANOVA.

Figure 4. Effect of caffeine on mephedrone-induced changes in the percentage time spent in the open arms (a), number of unprotected head dips (b) and number of stretch attend postures (c) on the elevated plus maze (EPM). Adult male Lister hooded rats (n=8) received i.p. saline vehicle (V, 1 ml/kg), mephedrone (Meph, 10 mg/kg), caffeine (Caff, 10 mg/kg) or combined mephedrone and caffeine (Meph+Caff, both 10 mg/kg) twice weekly on consecutive days for three weeks. EPM behaviours were measured on day eight, following the third injection. Data are displayed as mean ± SEM. *p<0.05, **p<0.01 compared to vehicle; ‡p<0.05 compared to mephedrone, Bonferroni multiple comparisons post-hoc following two-way ANOVA.

Figure 5. Effect of caffeine on mephedrone induced hypothermia. Adult male Lister hooded rats (n=8 per treatment group) received i.p. injection of either saline vehicle (1 ml/kg), mephedrone (Meph, 10 mg/kg), caffeine (Caff, 10 mg/kg) or a combination of caffeine and mephedrone (Caff+Meph, both 10 mg/kg) twice weekly on two consecutive days a week for three weeks. Rectal temperature was measured on day nine following the fourth challenge injection. Data are presented as mean ± SEM. **p<0.01, ***p<0.001 compared to vehicle; ‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001 combined mephedrone and caffeine compared to

mephedrone alone, Bonferroni multiple comparisons post-hoc following three-way repeated measures ANOVA.

Figure 6. Effect of mephedrone and caffeine administration on percent prepulse inhibition (PPI) of the acoustic startle response. Adult male Lister hooded rats (n=8 per treatment group) received i.p. injection of either saline vehicle (1 ml/kg), mephedrone (Meph, 10 mg/kg), caffeine (Caff, 10 mg/kg) or a combination of caffeine and mephedrone (Caff+Meph, both 10 mg/kg) twice weekly on two consecutive days a week for three weeks. PPI was measured on day 15 following the fifth challenge injection. Data are presented as mean ± SEM.

Figure 1

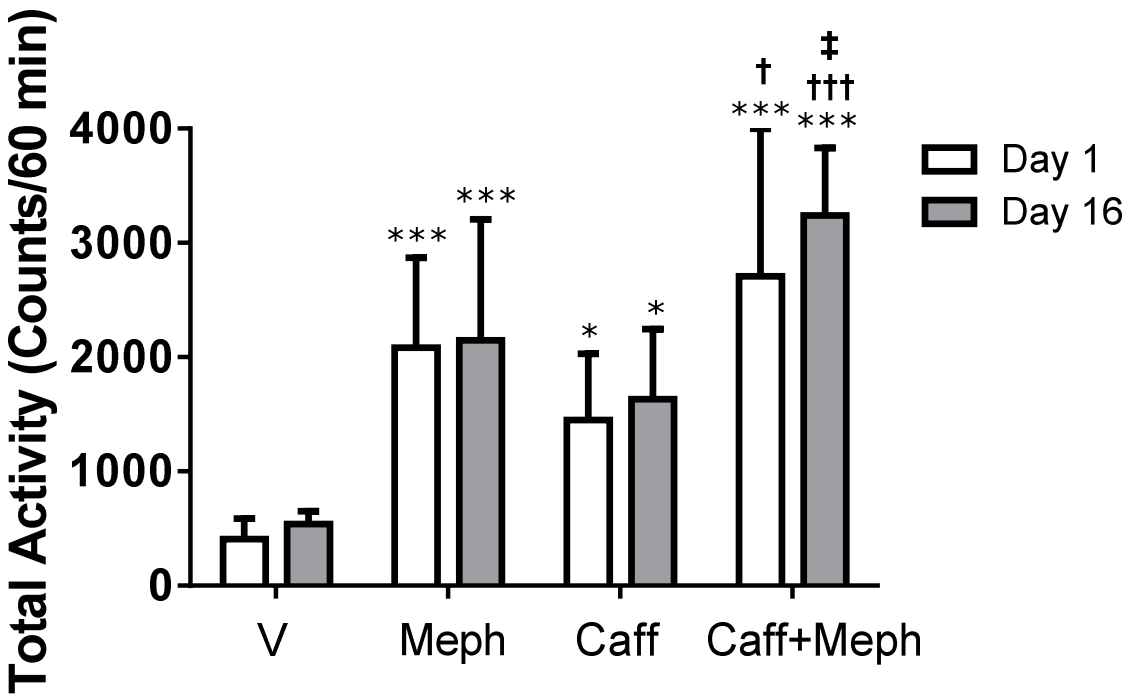
Total Activity (Counts/60 min)
4000
3000
2000
1000
0
V
Meph
Caff
Caff+Meph
Day 1
Day 16
***
***
*
*
***
†
***
‡
†††

Figure 2

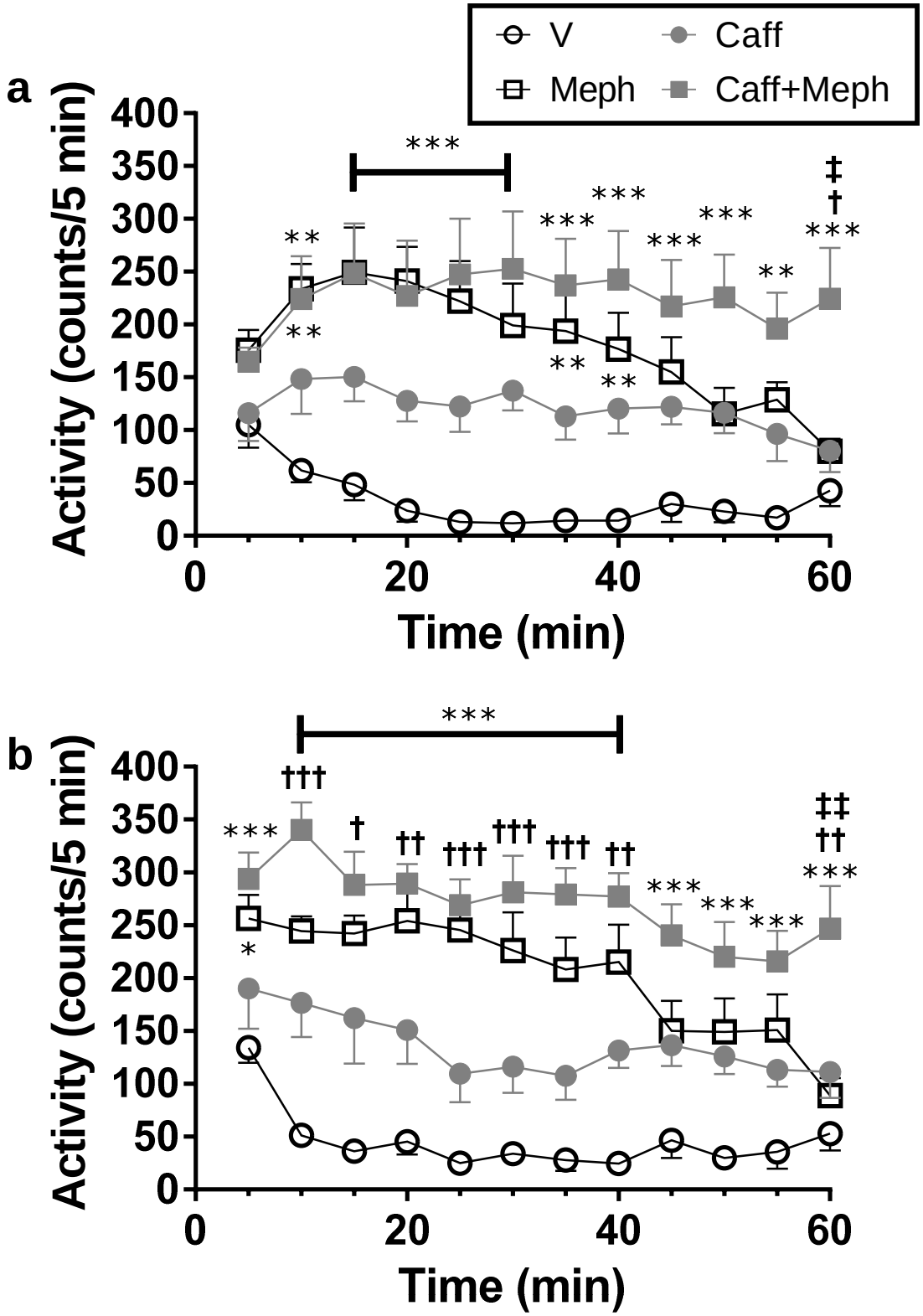
a
V
Caff
Meph
Caff+Meph
Activity (counts/5 min)
Time (min)
b
Activity (counts/5 min)
Time (min)

Figure 3

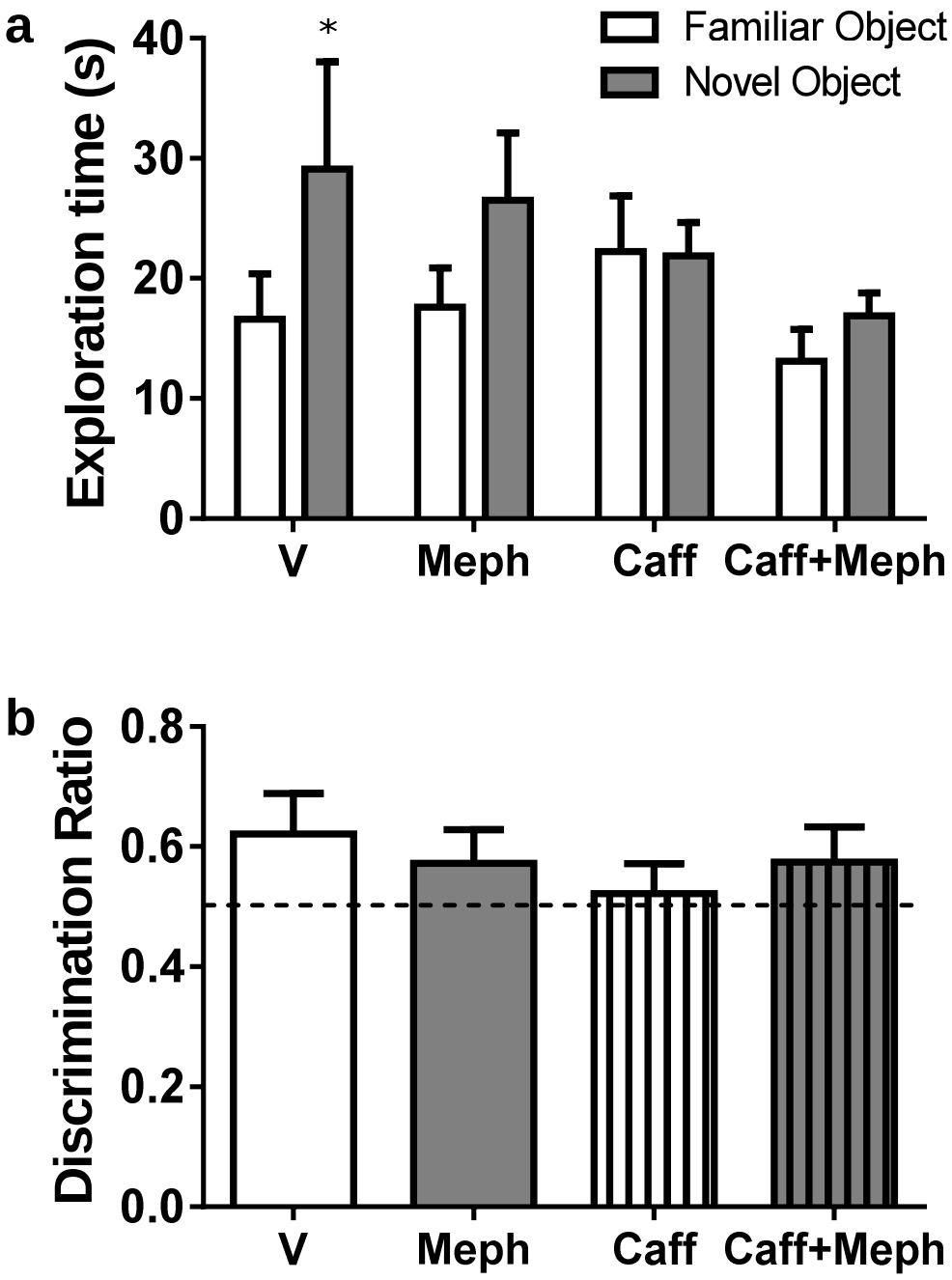
a
Exploration time (s)
40
30
20
10
0
*
Familiar Object
Novel Object
V
Meph
Caff
Caff+Meph
b
Discrimination Ratio
0.8
0.6
0.4
0.2
0.0
V
Meph
Caff
Caff+Meph

Figure 4

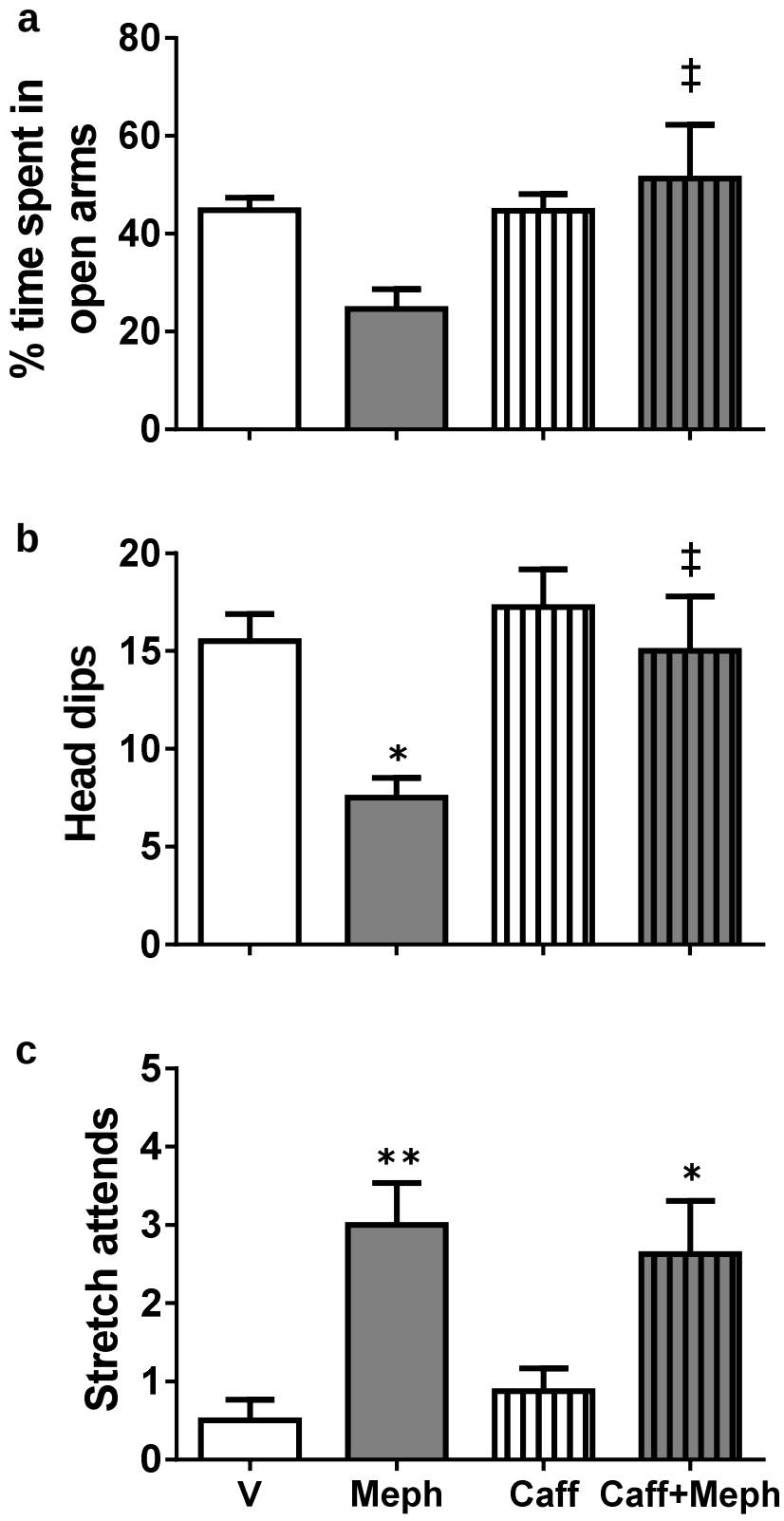
a
% time spent in open arms
80
60
40
20
0
‡
b
Head dips
20
15
10
5
0
*
‡
c
Stretch attends
5
4
3
2
1
0
**
*
V
Meph
Caff
Caff+Meph

Figure 5

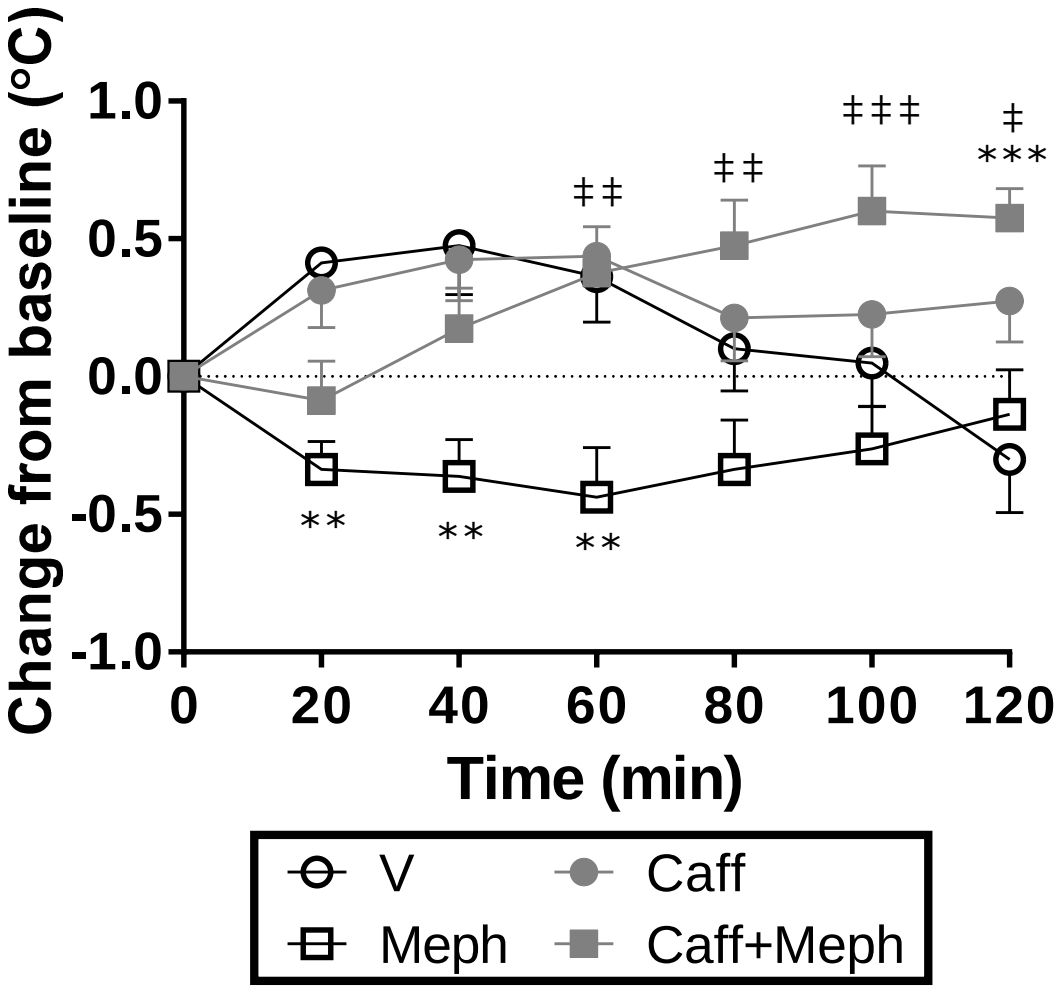
Change from baseline (°C)
1.0
0.5
0.0
-0.5
-1.0
0
20
40
60
80
100
120
Time (min)
**
**
**
‡‡
‡‡
‡‡‡
‡
***
V
Caff
Meph
Caff+Meph

Figure 6