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Contribution of serotonin and dopamine to changes in core body temperature and locomotor activity in rats following repeated administration of mephedrone

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Mephedrone users often employ repeated administration due to its short duration of action. This study investigated the role of dopamine and 5-HT in mephedrone-induced hyperactivity and hypothermia following repeated administration. Mephedrone increased striatal dopamine and 5-HT release following each injection. Mephedrone-induced hyperactivity and hypothermia were attenuated by 5-HT depletion, and by 5-HT_{1B} or 5-HT_{1A} receptor antagonism, respectively. These findings suggest that stimulation of central 5-HT release and/or inhibition of 5-HT reuptake plays a pivotal role in mephedrone-induced hyperactivity and hypothermia.

Abstract

The psychoactive effects of mephedrone are commonly compared to those of 3,4-methylenedioxymethamphetamine, but because of a shorter duration of action users often employ repeated administration to maintain its psychoactive actions. This study examined the effects of repeated mephedrone administration on locomotor activity, body temperature and striatal dopamine and 5-hydroxytryptamine (5-HT) levels, and the role of dopaminergic and serotonergic neurons in these responses. Adult male Lister hooded rats received three injections of vehicle (1ml/kg, i.p.) or mephedrone HCl (10mg/kg) at 2h intervals for radiotelemetry (temperature and activity) or microdialysis (dopamine and 5-HT) measurements. Intracerebroventricular pre-treatment (21 to 28 days earlier) with 5,7-dihydroxytryptamine (5,7-DHT, 150μg) or 6-hydroxydopamine (6-OHDA, 300μg) was used to examine the impact of 5-HT or dopamine depletion on mephedrone-induced changes in temperature and activity. A final study examined the influence of i.p. pre-treatment (-30min) with the 5-HT_{1A} receptor antagonist WAY-100635 (0.5mg/kg), 5-HT_{1B} receptor antagonist GR 127935 (3mg/kg) or the 5-HT_7 receptor antagonist SB-258719 (10mg/kg) on mephedrone-induced changes in locomotor activity and rectal temperature. Mephedrone caused rapid-onset hyperactivity, hypothermia (attenuated on repeat dosing), and increased striatal dopamine and 5-HT release following each injection. Mephedrone-induced hyperactivity was attenuated by 5-HT depletion and 5-HT_{1B} receptor antagonism, whereas the hypothermia was completely abolished by 5-HT depletion and lessened by 5-HT_{1A} receptor antagonism. These findings suggest that stimulation of central 5-HT release and/or inhibition of 5-HT reuptake play a pivotal role in both the hyperlocomotor and hypothermic effects of mephedrone, which are mediated in part via 5-HT_{1B} and 5-HT_{1A} receptors.

Keywords: 5-HT, dopamine, locomotor activity, mephedrone, microdialysis, telemetry

Introduction

The synthetic cathinone derivative 4-methylmethcathinone (mephedrone) was first synthesized in 1929 and became popular amongst recreational users at the beginning of the 21st century as a legal high (Green et al., 2014). Although mephedrone has been implicated in a number of deaths and became illegal in Europe and the United States between 2010 and 2012 (Dargan et al., 2011; Gershman and Fass, 2012), it remains widely available for illicit use (Elliott and Evans, 2014; Kelly et al., 2013; Yamamoto et al., 2013) and users report similar psychoactive effects to 3,4-methylenedioxymethamphetamine (MDMA). Mephedrone is a high-affinity substrate for the monoamine reuptake transporters for dopamine, noradrenaline and 5-hydroxytrypatamine (5-HT). Once transported into the cell mephedrone stimulates neurotransmitter release and disrupts vesicular storage by interaction with the vesicular monoamine transporter (VMAT) and can also stimulate non-exocytotic release by reversing the monoamine transporter flux (Simmler et al., 2013). Consistent with this, systemic mephedrone administration to freely moving rats elevates extracellular levels of dopamine, and to a greater extent 5-HT, in the nucleus accumbens (Baumann et al., 2012; Kehr et al., 2011; Wright et al., 2012).

Multiple re-dosing is common with mephedrone users attempting to maintain the desired effects of this short-acting drug, and while a typical recreational dose is often between 100-200mg, individuals may re-dose and ingest up to 4g in a single session (Schifano et al., 2011; Winstock et al., 2011). Most studies show the acute effect of a single injection, or self-administration of mephedrone in the rat is hypothermia (Aarde et al., 2013; Miller et al., 2013; Shortall et al., 2013a), but hyperthermia has also been reported following rapid repeated dosing (Baumann *et al*, 2012; Hadlock *et al*, 2011). Given the established association of hyperthermia with life-threatening adverse effects of MDMA (Docherty and Green, 2010), it is essential to see if there might be a similar adverse risk with repeated

mephedrone. The current study therefore examined the temporal profile of the temperature and locomotor response to short-term repeated mephedrone and established the involvement of serotonergic and dopaminergic neurons in these changes because of their known role in the effects of MDMA.

In the current study rats received three intraperitoneal (i.p.) injections of mephedrone (10mg/kg) at 2h intervals. Previous calculations suggest that this dose and route of mephedrone administration would produce similar plasma exposure to that occurring in many recreational users (Green et al., 2014). However, as pharmacokinetic studies of mephedrone have not been performed in man and there is wide variation in use of single or repeated recreational dose schedules, firm conclusions of the translatable accuracy of this dose cannot be made. Importantly, 10mg/kg i.p. produces robust but sub-maximal physiological and behavioral changes in the rat (Shortall et al., 2013a; Shortall et al., 2013b; Wright et al., 2012), thereby enabling detection of either enhanced or attenuated temperature and locomotor effects following repeated injection (Green et al., 2014). In the current study, all experiments were performed at ambient temperature as Wright et al, (2012) observed that mephedrone produced a comparable hypothermia and increase in locomotor activity when recorded at normal (23^{o}C) and elevated (27^{o}C) ambient room temperature in Wistar rats.

The current repeat dosing studies used continuous radiotelemetry to accurately and repeatedly record locomotor activity and core body temperature over a prolonged period in the same animal, without repeated insertion of a rectal probe, which would confound assessment of activity, at a consistent dose interval to previous preclinical studies using MDMA or mephedrone (Baumann et al., 2012; Baumann et al., 2008; Rodsiri et al., 2011). Because mephedrone causes hyperlocomotion (Shortall et al., 2013b) and the striatum plays a role in motor activity (Schultz, 2000), extracellular dopamine and 5-HT efflux from this

region were measured by *in vivo* microdialysis to examine whether neurotransmitter release correlated with the behavioral effects.

Previous pharmacological studies suggest the involvement of dopamine in mephedrone-induced hypothermia (Shortall et al., 2013a), so we further examined the contribution of serotonergic and dopaminergic neurons to the behavioral effects of mephedrone. Intracerebroventricular (i.c.v.) pre-treatment with selective neurotoxins (5,7-dihydroxytryptamine (5,7-DHT) and 6-hydroxytryamine, 6-OHDA, respectively) was used to determine the impact of 5-HT or dopamine depletion on the thermoregulatory and locomotor stimulant effects of repeated mephedrone measured using radiotelemetry. After identifying a role of 5-HT in mephedrone-induced hyperactivity and hypothermia, a final acute study investigated the involvement of specific 5-HT receptors by assessing the impact of selective 5-$HT_{1A,}$ 5-HT_{1B} or 5-HT_7 receptor antagonists on acute mephedrone-induced hyperlocomotion or hypothermia. These receptors were chosen because of their known role in locomotion and/or thermoregulation in the rat and to permit comparisons with the published effects of MDMA. Radiotelemetry was not used in these studies in accordance with the three Rs principle that invasive surgical implantation was unnecessary for acute measurement. This is the first study to concomitantly examine the effects of repeated mephedrone on hyperactivity, hypothermia and striatal dopamine efflux in short time periods (to provide a good temporal resolution) and establish the differential role of dopamine and 5-HT in mephedrone-induced hyperactivity and hyperthermia for comparison with the established effects of repeated MDMA injection.

Materials and Methods

Animals

Experimentally naïve young adult male Lister hooded rats (190-300g; Charles River UK) were used in all experiments. Rats were housed in groups of four prior to surgery and in individual cages post-surgery, under constant housing conditions (12 hours light:dark cycle with lights on at 07.00 hours, ambient temperature 21 ± 2˚C and relative humidity 55 ± 10%). Food and water were freely available, and wet mash was provided for five days post-surgery.

The drug doses and behavioral schedule used were chosen to comply with the three Rs of humane animal testing. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and Animal Research: Reporting of *In Vivo* Experiments guidelines with approval of University of Nottingham Local Ethical Committee.

Compounds

(±)-Mephedrone-HCl was purchased from Ascent Scientific, Cambridge, UK. Desipramine hydrochloride, ascorbic acid, 6-hydroxydopamine (6-OHDA) hydrobromide, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexanecarboxamide maleate (WAY-100635), N-[4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide hydrochloride (GR 127935) and (1R)-3,N-dimethyl-N-[1-methyl-3-(4-methylpiperidin-1-yl)propyl]benzenesulfonamide hydrochloride (SB-258719) were purchased from Tocris Bioscience, Bristol, UK. 5-7-dihydroxytryptamine (5,7-DHT) creatine sulphate was purchased from Sigma Aldrich, Dorset, UK. Mephedrone, desipramine, WAY-100635, GR 127935 and SB-258719 were dissolved in 0.154M saline, and 6-OHDA and 5,7-DHT were dissolved in 0.2% w/v ascorbic acid. All doses are quoted as the salt.

Radiotelemetry

Radiotelemetry was conducted as previously described (Rodsiri et al., 2011). Sterile radio-transmitters (Model TA 10TA-F20, DataScience International St Paul, MN, USA) were surgically implanted into the peritoneal cavity under isoflurane anesthesia. Post-operative analgesia was administered for 3 days (Rimadyl (carprofen), Pfizer; 4mg/kg, subcutaneous) and rats were allowed to recover for 9 days, then transferred to the procedure room 24 hours prior to testing to habituate. During testing, core body temperature and activity were continuously monitored in the home cage at ambient room temperature (19.9-20.9°C), using receivers (RPC-1) and A.R.T. v4 acquisition software (DataScience International, St Paul, MN, USA). Rats (n=5 per treatment group) received three i.p. injections of either saline vehicle (1ml/kg) or mephedrone (10mg/kg) at 2 hour intervals. Data were collected for 10 seconds every 2 minutes starting 60 minutes prior to the first injection, grouped into 20 minute epochs and expressed as mean ± standard error of the mean (SEM) activity counts. The change in body temperature (°C) from the baseline reading at 0 minute immediately prior to drug injection and the total cumulative activity counts in the 120 minutes following each injection were calculated and presented as mean ± SEM.

Microdialysis

As our Animals (Scientific Procedures) Act, 1986 project licence did not permit radiotelemetry and microdialysis to be performed in the same animal, microdialysis was measured in a separate cohort of rats using an identical protocol as previously described (Rodsiri et al., 2011). A CMA 12 polyurethane guide cannula (CMA Microdialysis AB, Kista, Sweden) was implanted above the striatum using stereotaxic coordinates anterior-posterior +0.48, medial-lateral ±3.0, dorsal-ventral -3.6 from Bregma (Paxinos and Watson, 1997) under isoflurane anesthesia. Seven days post-surgery, rats were briefly anaesthetized (isoflurane/O_2/N_2O) to insert a microdialysis probe (CMA12, 4mm polyarylethersulpone

membrane, 500µm outer diameter, 3µl internal volume with a 20kDa molecular cut-off; CMA Microdialysis AB) and each rat then placed in a circular arena (50cm diameter, 45cm height) with sawdust bedding, and food and water freely available. The probe was connected to a microinfusion pump (Harvard Apparatus, Holliston, MA, USA) using FEP tubing (Instech Laboratories Inc, Plymouth Meeting, PA, USA) via a liquid swivel (Instech 375/22, Instech Laboratories Inc, USA) to allow unrestricted movement and perfusion with artificial cerebrospinal fluid (125mM NaCl, 13.5mM $NaHCO_3$, 1.25mM KCl, 0.22mM NaH_2PO_4, 0.9mM Na_2HPO_4, 0.3mM Na_2SO_4, 0.5mM $MgCl_2$, 0.5mM $CaCl_2 2H_2O$, pH 7.4) at 1µl/min. The following day, rats (n=10 per treatment group) received three injections at 2 hour intervals of saline vehicle (1ml/kg) or mephedrone (10mg/kg i.p.), and samples were collected every 20 minutes into microtubes containing 5µl of 0.1M perchloric acid with 0.03% sodium metabisulfite. Samples were immediately stored on dry ice and then at -80°C until analysis by high performance liquid chromatography-electrical detection. After a collection of the final microdialysis sample, rats were euthanized with pentobarbital. Brains were rapidly removed and stored in 4% paraformaldehyde until sectioned (150µm coronal slices) using a vibrotome (Campden Instruments Ltd, Loughborough, UK). Location of the probe in the striatum was confirmed under a light microscope using Paxinos and Watson Rat Brain Atlas (1997).

Dopamine and 5-hydroxytrptamine depletion

In a third group of rats, bilateral i.c.v. injection of a monoamine neurotoxin (5,7-DHT or 6-OHDA) was performed under isoflurane anesthesia as previously described (King et al., 2009). All rats received desipramine (15mg/kg, i.p., 30 minute pre-treatment) to protect noradrenergic neurons prior to 5µl of 0.2% w/v ascorbic acid vehicle, 75µg/5µl of 5,7-DHT or 150µg/5µl of 6-OHDA into each lateral ventricle anterior-posterior -0.8, medial-lateral

±1.5, dorsal-ventral -3.8 from Bregma (Paxinos and Watson, 1997) at a rate of 5µl/minute. These doses were chosen as they reportedly produce a similar degree of depletion (70-75% below control value; King *et al*, 2009; Nowak *et al*, 2009). Twenty-one days post-surgery, each rat (*n*=8 per treatment group) received three injections of saline vehicle (1ml/kg) or mephedrone (10mg/kg i.p.) at 2 hour intervals, with radiotelemetry measurements as described previously. Using a cross-over design, rats received the opposite treatment during repeat monitoring 28 days post-surgery to minimize inter-individual responses to drug treatment or the lesion.

Neurochemical detection by high performance liquid chromatography-electrical detection

Seven days after radiotelemetry recording (35 days after i.c.v. injection), rats were killed by concussion followed by immediate decapitation, and the hypothalamus, right striatum, frontal cortex and hippocampus were collected on a refrigerated table (4°C), flash frozen in liquid nitrogen and stored at -80°C until analysis of dopamine, 5-HT and their major metabolites by high performance liquid chromatography-electrical detection, as previously described (Shortall et al., 2013b). Samples were thawed, weighed and sonicated for 30s in 800µl 0.05M perchloric acid containing 1µM sodium metabisulphite, centrifuged (17400x*g*, 4°C for 20min; Harrier 18/80: MSE Scientific Instruments, London, UK) and the supernatant filtered (0.45µM syringe tip filter, Kinesis Ltd, Saint Neots, UK). Monoamines were separated using a Targa C18 3µMVcolumn (100mm x 2.1mm; Phenomenex, Cheshire, UK) and detected using an Antec VT-03 cell with a glassy carbon 2mm working electrode set to +0.59V with an *in situ* Ag/AgCl ISAAC reference electrode. This system was also used to quantify extracellular dopamine and 5-HT in microdialysis samples. In addition, noradrenaline levels were measured in the same regions in 5,7-DHT and 6-OHDA pre-treated rats using a

modified HPLC protocol (a mobile phase of 20mM KH_2PO_4/Na acetate, 8mM KCl, 0.1mM EDTA, 1mM OSA, containing 10% methanol, pH 4.07).

Effect of 5-HT_{1A}, 5-HT_{1B} and 5-HT_7 receptor antagonists on mephedrone-induced hyperactivity and hypothermia following a single injection

Locomotor activity (LMA) and rectal temperature were recorded from separate groups to establish the role of specific 5-HT receptors in acute mephedrone-induced hyperactivity and hypothermia, using previously described methods (Shortall et al., 2013a; Shortall et al., 2013b). Rats (n=8 per treatment group) received saline vehicle (1ml/kg, i.p.), the 5-HT_{1A} receptor antagonist WAY-100635 (0.5mg/kg), the 5-HT_{1B} receptor antagonist GR 127935 (3mg/kg) or the 5-HT_7 receptor antagonist SB-258719 (10mg/kg), followed 30 minutes later by vehicle (1ml/kg, i.p.) or mephedrone (10mg/kg). Doses of 5-HT receptor antagonists were selected from previous studies (Fletcher et al., 2002; Graf et al., 2004; Guscott et al., 2003; Rusyniak et al., 2007).

Locomotor activity

Rats were placed in individual Perspex arenas and allowed to habituate for 60 minutes prior to the first injection. LMA was continuously recorded (in 5 minute time bins) for 30 minutes after the first and 60 minutes after the second injection using a Photobeam Activity System (San Diego Instruments, CA, USA) to record ambulation and rears.

Rectal temperature

In acute drug studies, rats were placed in individual Perspex arenas and basal temperature measured 40 minutes prior to the first injection to allow habituation to the recording procedure, which involved insertion of a rectal probe (Portec Instrumentation, Bedfordshire,

UK) to a depth of 6.5cm for approximately 20 seconds. Rectal temperature was measured immediately prior to each injection and then at 20 minute intervals for the next 2 hours.

Statistical Analysis

Analyses were performed using GraphPad Prism v6.02 or SPSS v21 software. Radiotelemetry data were analyzed by two-way repeated measures analysis of variance (ANOVA, with drug treatment and time as between and within factors, respectively) where rats received vehicle or mephedrone alone, or four-way repeated measures ANOVA (applied separately to 5,7-DHT and 6-OHDA groups, with i.c.v. injection and drug as between factors and time and week as within factors) where they also received i.c.v. injections. Dopamine microdialysis data were analyzed by two-way repeated measures ANOVA (with drug treatment and time as between and within factors, respectively). 5-HT microdialysis data were analyzed by one sample *t*-test against the pre-injection basal value as vehicle values fell below the limit of detection after 40 minutes. HPLC data were analyzed by one-way ANOVA where rats received vehicle or mephedrone alone, or two-way ANOVA where they also received i.c.v. injections. Acute LMA and rectal temperature data were analyzed by three-way repeated measures ANOVA (with 5-HT receptor antagonist pre-treatment and mephedrone treatment as between factors and time as the within factor). Total cumulative activity counts were analyzed by two-way ANOVA (with pre-treatment and treatment as between factors). Bonferroni multiple comparisons post-hoc test was used where appropriate and $P<0.05$ was considered statistically significant. All data are presented as mean ± SEM.

Results

Effects of repeated mephedrone on locomotor activity, body temperature and *in vivo* striatal dopamine release

Locomotor activity

Mephedrone increased activity above vehicle control levels for 40 minutes after the first injection and 80 minutes after the second and third injections, such that there was a drug x time interaction ($F_{(18,144)}$=3.43, P<0.001, Fig. 1a). The response to vehicle appeared to diminish with each consecutive administration, whereas the magnitude of the mephedrone-induced increase was similar after each injection.

Analysis of total cumulative activity in the 2 hours following each injection confirmed that mephedrone caused a reproducible hyperactivity on each occasion, with no significant difference between injections (First: 580±56; Second: 567±98; Third: 416±115 counts/2 hours). The peak response (increase compared with each pre-injection value) was also similar (7.2 ± 2.2; 8.2 ± 3.3; 7.9 ± 2.7). However, in vehicle-treated rats, the total decreased from 197±105 following the first to 61±15 after the third administration (P<0.05), suggesting some habituation to injection, which was not observed following mephedrone. This was reflected by a drug x injection number interaction ($F_{(2,16)}$=3.87, P<0.05), which was attributed to the change in the vehicle rather than mephedrone response.

Core body temperature

There were no between-group differences in temperature (recorded simultaneously with locomotor activity) in the 60 minutes prior to injection (data not shown), with baseline values (at the time of the first injection) being 37.8 ± 0.2°C in rats due to receive vehicle and 37.9 ± 0.1°C in those due to receive mephedrone. Following injection there was a drug x time interaction: ($F_{(18,144)}$=4.26, P<0.001, Fig. 1b), and although mephedrone decreased body

temperature to a greater extent than vehicle from 40-60 minutes after the first injection only, the maximum temperature change from baseline following each consecutive mephedrone injection was similar, being -1.3, -1.4 and -1.2°C following the first, second and third injections respectively. However, temperature did not return to baseline between injections, and the magnitude of each further decrease (compared with immediate pre-injection values; at T_0, T_{120}, T_{240}) was attenuated (First: -1.3 ± 0.3°C; Seconds: -0.6 ± 0.3°C; Third: -0.2 ± 0.2°C reaching significance for the last injection; $P<0.05$ from the first response) suggesting tolerance occurred.

In vivo striatal dopamine and 5-hydroxydopamine efflux

In a separate group of rats to those used for radiotelemetry, there were no between-group differences in basal extracellular dopamine levels in the 60 minutes prior to the first injection (7.32 ± 1.65pmol/ml in rats due to receive vehicle and 5.08 ± 0.85pmol/ml in those due to receive mephedrone). Following injection, there was a drug x time interaction ($F_{(18,319)}=3.55$, $P<0.001$, Fig. 1c). Mephedrone rapidly increased extracellular dopamine levels above vehicle control for 40 minutes after the first and third injections and 60 minutes after the second, but dopamine levels returned to near basal between injections. Thus, each injection produced a similar magnitude (First: 298%; Second: 520%; Third: 435% peak change from baseline) and time course of elevation in extracellular striatal dopamine.

Basal extracellular levels of 5-HT were close to the detection limit but equivalent in both groups when measured immediately prior to the first injection (0.295 ± 0.12 and 0.323 ± 0.07pmol/ml in control and mephedrone groups, respectively). In vehicle-treated rats, post-injection 5-HT levels remained either close to or below the detection limit, and the pre-injection value has been used to calculate the percentage increase (Fig. 1d). The first mephedrone injection failed to elevate extracellular 5-HT, but the two subsequent injections

produced statistically significant increases ($P<0.05$ to $P<0.01$, versus mean baseline 60 minutes after the second [458% peak change from baseline] and 40 minutes after the third injection [351% peak change from baseline]).

Ex vivo monoamine content

There was no significant effect of repeated mephedrone administration on tissue levels of dopamine, 5-HT or their major metabolites in the hypothalamus, striatum, hippocampus or frontal cortex measured 7 days after radiotelemetry recording (data not shown).

Effects of 5-HT or dopamine depletion on repeated mephedrone-induced changes in locomotor activity and core body temperature

Locomotor activity

The third experiment again found that mephedrone caused a rapid increase in locomotor activity, which returned to basal levels between injections, whereas vehicle produced only a very small transient response in the same rats. Four-way repeated measures ANOVA confirmed a drug x 5,7-DHT ($F_{(1,28)}=4.92$, $P<0.001$) and drug x time interactions ($F_{(18,504)}=9.32$, $P<0.001$), but no drug x 5,7-DHT x time interaction ($F_{(18,504)}=1.24$, $P>0.05$, Fig. 2a). Of note, 5,7-DHT pre-treatment attenuated the mephedrone-induced hyperactivity, such that there was no significant response of 5,7-DHT-treated rats to the first mephedrone injection and responses to the second and third mephedrone injections were significantly lower in 5,7-DHT-treated rats than sham controls (while the response to vehicle was unaffected). In contrast, 6-OHDA lesion did not alter mephedrone-induced hyperactivity (drug x 6-OHDA x time: $F_{(1,28)}=1.66$, $P>0.05$, Fig. 2b).

Consistent with the previous experiment, total cumulative activity in the 2 hours following each injection confirmed mephedrone-induced hyperactivity, with a drug x

injection number interaction ($F_{(2,36)}$=5.31, P<0.01, Table 1). 5,7-DHT completely prevented the response to the first mephedrone injection and attenuated that to the third (P<0.01versus sham control mephedrone response). In contrast, 6-OHDA-treated rats continued to exhibit an increase in cumulative activity following each mephedrone injection which did not differ from the response in mephedrone-treated sham controls.

Core body temperature

Basal core body temperatures prior to the first injection on each test day (recorded simultaneously with locomotor activity in the same sham and lesioned rats) were equivalent, being 37.2 ± 0.2 and 37.5 ± 0.2°C in sham controls, 36.9 ± 0.2 and 37.2 ± 0.2°C in 5,7-DHT and, 37.5 ± 0.2 and 37.4 ± 0.2°C in 6-OHDA rats prior to injection of vehicle or mephedrone, respectively. The maximum temperature change from baseline following each mephedrone injection in sham controls (-1.2, -1.2 and -1.0°C following the first, second and third injections, respectively) was equivalent, but in agreement with the first study, the maximum temperature decrease (compared with pre-injection value) was attenuated following both the second and third (P<0.05) compared with the first injection (First: -1.2 ± 0.2°C; Second: -0.3 ± 0.1°C; Third: -0.1 ± 0.2°C).

There was a main drug x 5,7-DHT x time interaction ($F_{(18,504)}$=1.72, P<0.05; Fig. 2c,d) such that mephedrone significantly reduced core body temperature in sham controls 20-80 minutes after the first, 20-60 minutes after the second and at 40 and 80 minutes after the third injection. 5,7-DHT pre-treatment completely abolished mephedrone-induced hypothermia, and there was no difference between vehicle and mephedrone-treated 5,7-DHT rats at any time point, and the temperature change in 5,7-DHT mephedrone-treated rats was significantly attenuated compared to mephedrone-treated sham controls. There was also a main drug x 6-OHDA x time interaction ($F_{(18,504)}$=1.76, P<0.05), such that mephedrone-

induced hypothermia was reduced in 6-OHDA pre-treated rats where the decrease in temperature was only significant from sham controls at 40-80 minutes and at 40 minutes following the first and second injections, respectively.

Ex vivo neurochemistry

Dopamine, 5-HT and noradrenaline levels in the hypothalamus, right frontal cortex, hippocampus and striatum were measured 35 days after neurotoxin administration to confirm selective monoamine depletion. As expected the serotonergic neurotoxin, 5,7-DHT, significantly reduced 5-HT to 46% of control in the frontal cortex ($P<0.001$), 13% in the hippocampus ($P<0.01$), 42% in the hypothalamus ($P<0.001$) and 66% in the striatum although the latter did not reach significance due to high individual variation (Table 2). In contrast, 6-OHDA reduced dopamine to 52% of control in the striatum ($P<0.001$) and 56%, 80% and 86% of control in the frontal cortex, hippocampus and hypothalamus, respectively, although the depletion in these areas was not statistically significant (Table 2). However, the 6-OHDA-induced decrease in striatal dopamine was accompanied by a significant reduction in hippocampal 5-HT ($F_{(2,20)}=7.19$, $P<0.01$) as well as decreased noradrenaline levels in the hypothalamus and hippocampus ($F_{(2,21)}=9.53$, $P<0.001$), but noradrenaline levels were unchanged in the other regions examined.

Effect of 5-HT_{1A}, 5-HT_{1B} and 5-HT_7 receptor antagonists on acute mephedrone-induced hyperactivity and decreases in rectal temperature

In a final study, separate groups of rats were pre-treated i.p. with the 5-HT_{1A} receptor antagonist, WAY-100635, the 5-HT_{1B} receptor antagonist, GR 127935, or the 5-HT_7 receptor antagonist, SB-258719 to investigate the role of specific 5-HT receptors in mephedrone-induced hyperactivity and hypothermia.

Locomotor activity

None of the three 5-HT receptor antagonists had any effect on activity counts following their injection (data not shown). The predominant locomotor stimulant effect of mephedrone in vehicle pre-treated rats was a prolonged increase in ambulatory activity ($p<0.05$-0.001, accompanied by a smaller increase in fine movement without increased rearing consistent with previous studies by our group (Shortall et al., 2013b) and the current telemetry data. It was briefly attenuated WAY-100653 (Fig. 3a) at 15 minutes post-injection and more substantially attenuated by GR 127935 (Fig. 3b) from 15-35 minutes post-injection, but completely unaffected by SB-258719 (Fig. 3c). Consistent with the time-course data, total cumulative ambulation in the 60 minutes post-mephedrone period was reduced from 1244±199.1 in vehicle pre-treated rats to 706.8±78.4 by GR 127935 pre-treatment (pre-treatment x mephedrone interaction: $F_{(1,28)}=5.39$, $p<0.05$), but unaffected by WAY-100653 (1007±129.6) or SB-258719 (1557±176.7).

Rectal temperature

Injection of vehicle had no effect on rectal temperature irrespective of whether rats were pre-treated with WAY-100635 (Fig. 4a), GR 127935 (Fig. 4b) or SB-258719 (Fig. 4c). Mephedrone caused a transient but significant decrease in rectal temperature at 20 minutes ($P<0.001$) and 40 minutes ($P<0.05$) post-injection compared with vehicle, which was consistent with the duration and magnitude observed in a previous study by our group (Shortall et al., 2013a). There was a WAY-100635 x mephedrone x time interaction ($F_{(7,196)}=2.84$, $P<0.01$, Fig. 4a), such that the mephedrone-induced hypothermia was partially blocked by WAY-100635 from 20-40 minutes post-mephedrone. However, there was no

significant GR 127935 x mephedrone x time interaction ($F_{(7,196)}$=1.16, P>0.05, Fig. 4b) nor a SB-258719 x mephedrone x time interaction ($F_{(7,196)}$=1.35, P>0.05, Fig. 4c).

Discussion

This study investigated the effects of repeated mephedrone injection on core body temperature, locomotor activity and striatal dopamine and 5-HT release in the rat, and examined the role of dopamine and 5-HT containing neurons on mephedrone-induced changes in body temperature and activity. This is one of only a few studies to use radiotelemetry to obtain a high temporal resolution of changes appropriate for the short-duration responses (Aarde et al., 2013; Miller et al., 2012; Wright et al., 2012). The main findings were as follows; (1) while hyperactivity and increase in extracellular striatal dopamine seen after the first mephedrone injection were similar in magnitude and time course to those seen following the second and third injections, the hypothermia was attenuated with repeated dosing; (2) extracellular striatal 5-HT overflow was more variable but was enhanced when second and third injections were given when compared with the first response; (3) 6-OHDA did not affect hyperactivity but reduced the duration of the hypothermic response; (4) 5,7-DHT administration and 5-HT_{1B} receptor antagonism attenuated mephedrone-induced hyperactivity; (5) 5,7-DHT administration completely abolished, and 5-HT_{1A} receptor antagonism attenuated mephedrone-induced hypothermia. Importantly, some of these observed effects contrast with those reported with MDMA, suggesting differing possible adverse effects following recreational use.

Mephedrone has a high affinity for rat dopamine and 5-HT transporters as well as the 5-HT_{2A} and 5-HT_{2C} receptors, and α_{1A}- and α_{2A}-adrenoceptors (Eshleman et al., 2013; Martinez-Clemente et al., 2012; Simmler et al., 2013). It increases extracellular dopamine and to an even greater extent 5-HT in the nucleus accumbens (Baumann et al., 2012;

Eshleman et al., 2013; Kehr et al., 2011; Wright et al., 2012). The current study, in contrast, suggests that in the striatum, the percentage increase in 5-HT and dopamine is rather similar, at least after the second and third injections.

The repeated dose given in the current study (10mg/kg) did not produce any neurotoxic loss of brain regional dopamine or 5-HT measured 7 days post-injection. This is in marked contrast to MDMA, where a repeated dose schedule that releases striatal dopamine also produces significant long-term neurotoxic 5-HT depletion in the rodent (Green et al., 2003), but similar to methcathinone where a much larger dose than that needed to elicit behavioral changes is required to obtain neurotoxicity (Sparago et al., 1996). Although hypothermia protects against MDMA neurotoxicity (Malberg and Seiden, 1998), a previous study in which mephedrone produced hyperthermia in the rat also failed to detect any neurotoxic loss of post-mortem brain monoamines two weeks after a repeated dosing schedule similar to that used in the current study (Baumann et al., 2012). These data therefore suggest that rapid repeated mephedrone administration is less likely to produce monoamine neurotoxicity than MDMA.

Mephedrone induces hyperactivity in rodents following both acute and intermittent administration (Angoa-Perez et al., 2012; Baumann et al., 2012; Kehr et al., 2011; Marusich et al., 2012; Shortall et al., 2013b; Wright et al., 2012). Mephedrone (0.5 to 30 mg/kg i.p. or subcutaneous) has consistently been shown to elicit hyperactivity in a variety of rat strains, when given during both the light (Gregg et al., 2013; Lisek et al., 2012; Shortall et al., 2013b) or dark (Miller et al., 2013; Motbey et al., 2012) phase of the circadian cycle. Because significant hyperactivity was found irrespective of circadian phase, the current study was conducted in the light phase to enable comparison with the many studies on MDMA, including our own, which use this protocol. In the current study, repeated 'binge-style' mephedrone administration caused reproducible hyperactivity after each injection, the onset

of which occurred within minutes of injection but returned to baseline levels within 1 hour. The time courses for both the striatal dopamine release and the hypothermia are consistent with a previous study using a single systemic injection (Shortall et al., 2013b). It is noteworthy that the peak plasma level of mephedrone in the rat follows a similar temporal pattern following subcutaneous injection (Miller et al., 2013). Importantly, the total ambulatory activity counts following the second and third injections of mephedrone were comparable to those following the first injection. This response therefore differs markedly from MDMA where progressively increasing hyperactivity was observed following a similar repeated dosing schedule (Rodsiri et al., 2011).

In the current study, central 5-7-DHT administration markedly attenuated the hyperactivity observed following mephedrone injection 21 or 28 days later, while i.c.v. injection of 6-OHDA had no effect on mephedrone-induced hyperactivity. This observation is consistent with the ability of pCPA-induced 5-HT depletion to reduce mephedrone-induced hyperactivity in mice (Lopez-Arnau et al., 2012) and supports a key role for 5-HT in mephedrone-induced hyperactivity. In the current study blockade of 5-HT_{1B}, and to a lesser extent 5-HT_{1A} (but not 5-HT_7), receptors also reduced mephedrone-induced hyperactivity, and this is consistent with similar observations on MDMA-induced hyperactivity (Fletcher et al., 2002; McCreary et al., 1999). The affinity of mephedrone for the 5-HT_{1B} receptor has not yet been investigated and so it is difficult to ascertain whether this effect on mephedrone-induced hyperactivity is due to a direct effect on this receptor.

Although hyperthermia has not been recorded in mephedrone users, there is evidence that it alters peripheral thermoregulation because reported adverse effects include cold/blue fingers, hot flushes and sweating (Winstock et al., 2011; Wood and Dargan, 2012) which may occur from peripheral changes in blood flow. Earlier studies have generally failed to observe hyperthermia in rodents given an acute injection of mephedrone, even when the animals are

group-housed or kept in raised ambient temperature (Shortall et al., 2013a; Wright et al., 2012). However, hyperthermia was observed in two studies investigating the effects of repeated mephedrone injection (Baumann et al., 2012; Hadlock et al., 2011). Of note, both of these studies used Sprague Dawley rats and subcutaneous injections so there could be strain and/or pharmacokinetic differences (Wright et al., 2012). These repeated injection studies also used a rectal probe to measure the response at 1 hour intervals so the observed hyperthermia may have resulted from an additive effect of repeated mephedrone injection combined with stress-induced hyperthermia associated with rectal measurement as evident in vehicle control animals (Baumann et al., 2012; Hadlock et al., 2011). The current study therefore used radiotelemetry to measure the temperature response following repeated mephedrone injection and showed it produced hypothermia as reported following a single injection (Aarde et al., 2013; Miller et al., 2012). In the current study, 5,7-DHT administration abolished the hypothermic response to mephedrone. Furthermore, administration of the 5-HT_{1A} receptor antagonist, WAY-100635, attenuated mephedrone-induced hypothermia while antagonism of the 5-HT_{1B} or 5-HT_7 receptors had no effect. Although 5-HT_{1A} receptors are implicated in the hypothermic response, their involvement is almost certainly a consequence of 5-HT release and/or inhibition of reuptake, because the low affinity of mephedrone for the 5-HT_{1A} receptor (Ki>20μM; Simmler et al, 2013) makes any direct effect unlikely. Interestingly, pre-treatment with WAY-100635 at the same dose as used herein also prevents the hypothermic response to MDMA (Rusyniak et al., 2007) but the involvement of 5-HT_{1B} and 5-HT_7 receptors in the thermoregulatory effect of MDMA has not been documented. In contrast, mephedrone injection to 6-OHDA-treated rats produced a hypothermic response which was shorter in duration than that seen in sham controls. At first, this appears paradoxical because we have shown that administration of the dopamine D_1 receptor antagonist, SCH 23390, prolonged mephedrone-induced hypothermia (Shortall et al.,

2013a). However, the limited depletion of dopamine in the hypothalamus makes it difficult to come to any firm conclusion about the role of dopamine in mephedrone-induced hypothermia.

Tolerance to the hypothermic effect of mephedrone is intriguing and is unlikely to be due to a pharmacokinetic effect as locomotor and dopamine responses were unaffected. Considerable evidence shows that 5-HT plays a major role in thermoregulation, particularly when body temperature is perturbed by amphetamine-like drugs (Docherty and Green, 2010). However, it is unclear whether increased 5-HT release with repeated dosing, observed herein, is associated with tolerance to the hypothermic effect of mephedrone. Although the limited depletion of hypothalamic dopamine makes it impossible to completely exclude a role of this monoamine in mephedrone-induced hypothermia the fact that this response is unaffected by dopamine D_2 receptor blockade and prolonged by D_1 receptor antagonism (Shortall et al., 2013a) suggests that mephedrone-induced increases in dopamine efflux are unlikely to contribute to the drug-induced hypothermia. In contrast, modulation of central serotonergic neurotransmission plays a key role in mediating both the hyperlocomotor and hypothermic effects of mephedrone.

Although caution is required in attempting to translate the relevance of these findings in the rat to those in man, they demonstrate the need to evaluate the pharmacology and psychoactive effects of any new amphetamine analogues and not rely on prediction from structural analogy.

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Disclosure

There is no conflict of interest to report.

Authors Contribution

SES, ARG, KCFF and MVK were responsible for the study concept and design. SES, CHS and MVK performed surgical procedures. SES, FJPE and MVK contributed to the acquisition of animal data. SES and MVK drafted the manuscript. ARG, KCFF and MVK provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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Table 1 Total activity counts following repeated mephedrone administration to 5-HT or dopamine depleted rats

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

ANOVA = analysis of variance; SEM = standard error of the mean; 5-HT = 5-hydroxytrptamine; 5,7-DHT = 5,7-dihydroxytrptamine; 6-OHDA = 6-hydroxydopamine. Horizontal ambulatory counts (mean ± SEM) were measured following each of three i.p. injections of saline vehicle (V, 1ml/kg) or mephedrone (Meph, 10mg/kg) at 2 hour intervals, 21 or 28 days after bilateral i.c.v. injection under isoflurane anesthesia of either 0.2% ascorbic acid vehicle (5µl), 5,7-DHT (75µg/5µl per side) or 6-OHDA (150µg/5µl per side), to individually housed adult male Lister hooded rats (n = 8 per treatment group). ***P<0.001, **P<0.01 sham + mephedrone compared to sham + vehicle; ***P< 0.001, **P< 0.01 lesion + mephedrone compared to sham + vehicle, †††P<0.001, †P<0.05 lesion + mephedrone compared to lesion + vehicle, ‡‡P<0.01 lesion + mephedrone compared to sham + mephedrone, †P<0.05 compared to the first injection of the same treatment group, Bonferroni multiple comparisons *post hoc* following three-way repeated measures ANOVA. Of note, 5,7-DHT reduced the hyperactivity produced by mephedrone (it was no longer greater than the effect of vehicle) while 6-OHDA did not attenuate the activity response to mephedrone.

Table 2 Effect of i.c.v. administration of 6-OHDA or 5,7-DHT on brain tissue dopamine, 5-HT and noradrenaline levels 5 weeks post-surgery

	Tissue levels (pmol/mg)			
Lesion Type	*Frontal cortex*	*Hippocampus*	*Hypothalamus*	*Striatum*
Dopamine				
Sham	0.72 ± 0.2	0.4 ± 0.03	3.0 ± 0.2	55.6 ± 4.5
5,7-DHT	0.46 ± 0.02	0.4 ± 0.02	3.4 ± 0.2	60.4 ± 2.2
6-OHDA	0.40 ± 0.02	0.3 ± 0.01	2.5 ± 0.2	28.6 ± 5.5***
5-HT				
Sham	3.5 ± 0.3	3.8 ± 0.4	7.2 ± 0.5	4.4 ± 0.3
5,7-DHT	1.6 ± 0.3***	0.5 ± 0.1***	3.0 ± 0.4***	2.9 ± 0.5
6-OHDA	4.0 ± 0.2	1.9 ± 0.6*	5.4 ± 0.6	5.5 ± 0.5
Noradrenaline				
Sham	1.8 ± 0.1	2.8 ± 0.3	16.8 ± 1.5	0.9 ± 0.1
5,7-DHT	1.9 ± 0.1	2.2 ± 0.5	15.9 ± 1.5	1.1 ± 0.2
6-OHDA	1.7 ± 0.1	0.9 ± 0.2**	8.5 ± 1.4**	1.0 ± 0.3

ANOVA = analysis of variance; SEM = standard error of the mean; 5-HT = 5-hydroxytrptamine; 5,7-DHT = 5,7-dihydroxytrptamine; 6-OHDA = 6-hydroxydopamine. Dopamine, 5-HT and noradrenaline levels (mean ± SEM, pmol/mg wet weight) were measured 35 days after bilateral i.c.v. injection of either 0.2% ascorbic acid vehicle (5µl per side), 5,7-DHT (75µg/5µl per side) or 6-OHDA (150µg/5µl per side) to individually housed male Lister hooded rats (n=8 per treatment group). ***P<0.001, **P<0.01, *P<0.05 compared to sham controls, Bonferroni *post hoc* following one-way ANOVA. Note that 5,7-DHT selectively reduced 5-HT in the frontal cortex, hippocampus and hypothalamus while 6-OHDA depleted dopamine in the striatum without affecting noradrenaline and 5-HT in this region.

Fig. 1. Comparison of the effect of repeated injection of saline vehicle (V, 1ml/kg i.p., n=5) or mephedrone (Meph, 10mg/kg, n=5) on (a) locomotor activity, (b) core body temperature and *in vivo* extracellular striatal (c) dopamine and (d) 5-HT levels (n=10 per treatment group) in individually housed adult male Lister hooded rats. Vehicle or mephedrone were injected once every 2 hours at 0, 120 and 240 minutes (as indicated by the arrows). Temperature data are represented as change from baseline (t = 0 min, °C). All data are presented as mean ± SEM and (a-c) *P<0.05, **P<0.01, ***P<0.001 compared to vehicle, Bonferroni multiple comparisons post-hoc following two-way repeated measures ANOVA. (d) 5-HT levels fell below the detection limit in most rats at more than 40 minutes after starting microdialysis collection preventing data for subsequent time points to be displayed so the change in 5-HT in the mephedrone group was analyzed against the pre-injection basal value (0.350 ± 0.091pmol/ml) using one sample t test, **P<0.01. For clarity of presentation, microdialysis data (c,d) are displayed as percentage change from baseline, but statistical analysis was performed on the raw data.

Fig. 2. Effects of bilateral i.c.v. injection (5µl per side) of 0.2% ascorbic acid vehicle, 5,7-DHT (75µg/5µl per side) or 6-OHDA (150µg/5µl per side, b, d) pre-treatment on saline vehicle (1ml/kg) or mephedrone (10mg/kg) induced change in (a, b) ambulatory activity counts and (c,d) core body temperature change from baseline (at t = 0 min, °C) in adult male Lister hooded rats (n=8 per treatment group). Using a cross over design, each rat received vehicle or mephedrone on day 21, and the opposite treatment 28 days post-surgery. Vehicle and mephedrone were injected once every two hours at 0, 120 and 240min (as indicated by the arrows). All data are presented as mean ± SEM. For clarity 5,7-DHT and 6-OHDA have been presented as separate Fig.s versus the sham controls but ANOVA has been performed on all groups. *P<0.05, **P<0.01, ***P<0.001 sham mephedrone compared to sham +

vehicle ; ***P< 0.001, **P< 0.01, *P<0.05 lesion mephedrone compared to sham vehicle, †††P<0.001, ††P<0.01, †P<0.05 lesion mephedrone compared to lesion vehicle, ‡‡‡P<0.001, ‡‡P<0.01, ‡P<0.05 lesion mephedrone compared to sham mephedrone, Bonferroni multiple comparisons post-hoc following four-way repeated measures ANOVA.

Fig. 3. Comparison of the effect of (a) the 5-HT_{1A} receptor antagonist WAY-100635, (b) the 5-HT_{1B} receptor antagonist GR 127935 and (c) the 5-HT_7 receptor antagonist SB-258719 on saline vehicle (1ml/kg) or mephedrone (10mg/kg)-induced change in locomotor activity following a single injection in adult male Lister hooded rats (n=8 per treatment group). Saline vehicle (1ml/kg), WAY-100635 (0.5mg/kg), GR 127935 (3mg/kg) or SB-258719 (10mg/kg) was injected -30 minutes, before saline or mephedrone at time = 0 min. All data are presented as mean ± SEM Line indicates significance at indicated time points. *P<0.05, ***P<0.001 vehicle mephedrone versus vehicle vehicle; †P<0.05, ††P<0.01, †††P<0.001 antagonist mephedrone versus vehicle vehicle; †P<0.05, ††P<0.01, †††P<0.001 antagonist mephedrone versus antagonist vehicle, ‡P<0.05, ‡‡P<0.05 antagonist mephedrone versus vehicle mephedrone, Bonferroni post-hoc following three-way repeated measures ANOVA.

Fig. 4. Comparison of the effect of (a) the 5-HT_{1A} receptor antagonist WAY-100635, (b) the 5-HT_{1B} receptor antagonist GR 127935 and (c) the 5-HT_7 receptor antagonist SB-258719 on saline vehicle (1ml/kg) or mephedrone (10mg/kg) induced change in rectal temperature following a single injection in adult male Lister hooded rats (n=8 per treatment group). Saline vehicle (1ml/kg), WAY-100635 (0.5mg/kg), GR 127935 (3mg/kg) or SB-258719 (10mg/kg) were injected -30 minutes before saline or mephedrone at time = 0 min. Rectal temperature was measured at -30 minutes and at 20 minute intervals from 0 to 120 minutes, and data are expressed as change in temperature (°C, mean ± SEM) from the reading taken at

0 minute. ***$P<0.001$ vehicle mephedrone versus vehicle vehicle; †$P<0.05$, †††$P<0.001$ antagonist mephedrone versus vehicle vehicle; †$P<0.05$, ††$P<0.01$, †††$P<0.001$ antagonist mephedrone versus antagonist vehicle, Bonferroni post-hoc following three-way repeated measures ANOVA.

Figure 1

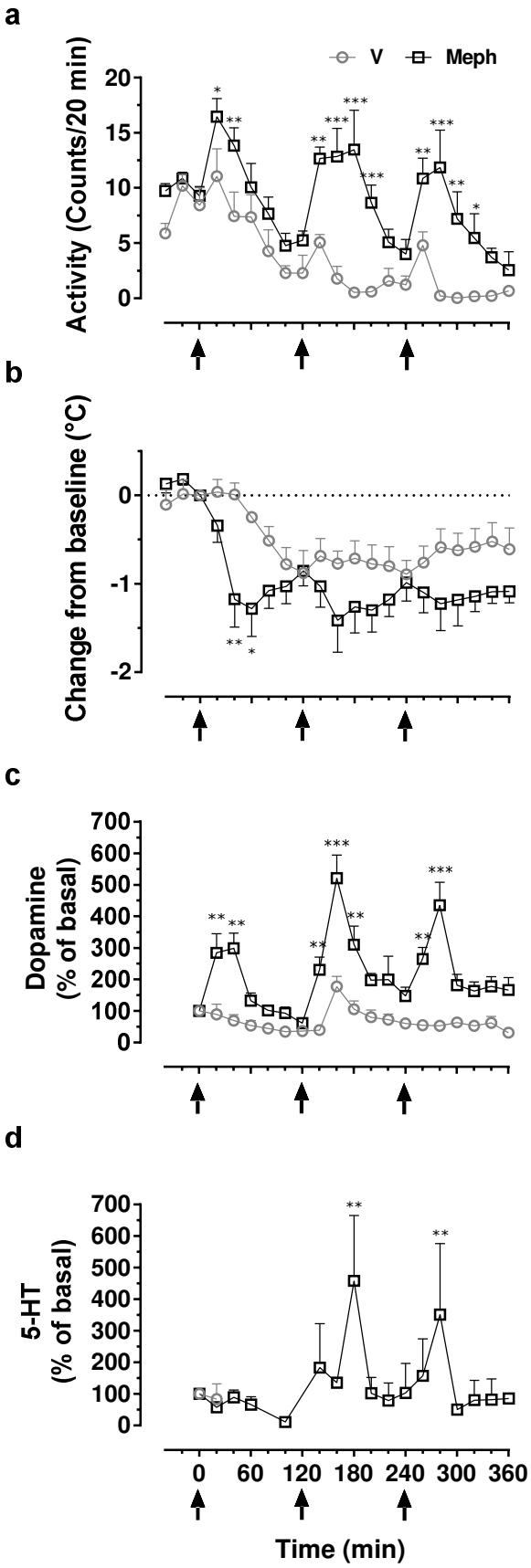

a
V
Meph
Activity (Counts/20 min)
20
15
10
5
0
b
Change from baseline (°C)
0
-1
-2
c
Dopamine
(% of basal)
700
600
500
400
300
200
100
0
d
5-HT
(% of basal)
700
600
500
400
300
200
100
0
0
60
120
180
240
300
360
Time (min)

Figure 2

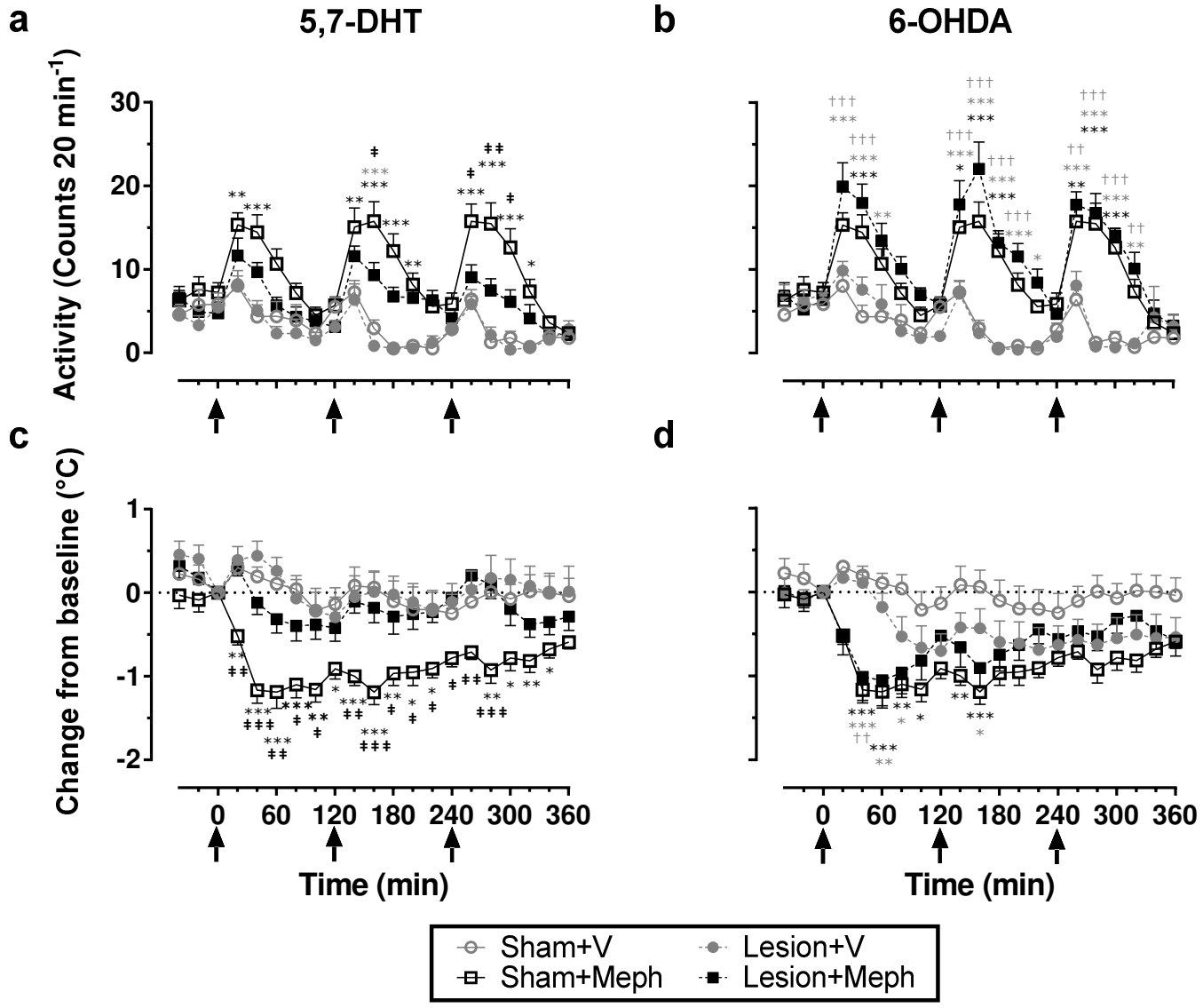

a
5,7-DHT
b
6-OHDA
Activity (Counts 20 min-1)
30
20
10
0
c
d
Change from baseline (°C)
1
0
-1
-2
0
60
120
180
240
300
360
Time (min)
Sham+V
Sham+Meph
Lesion+V
Lesion+Meph

Figure 3

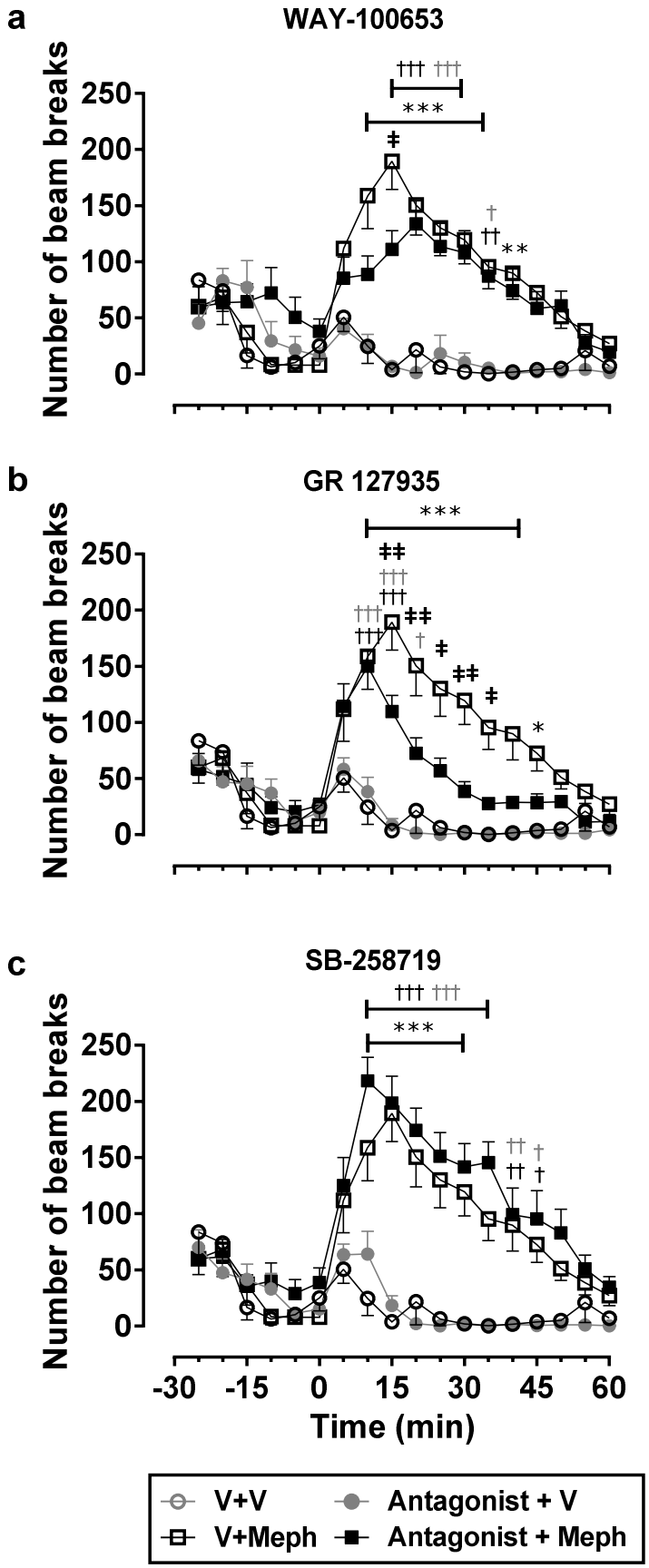
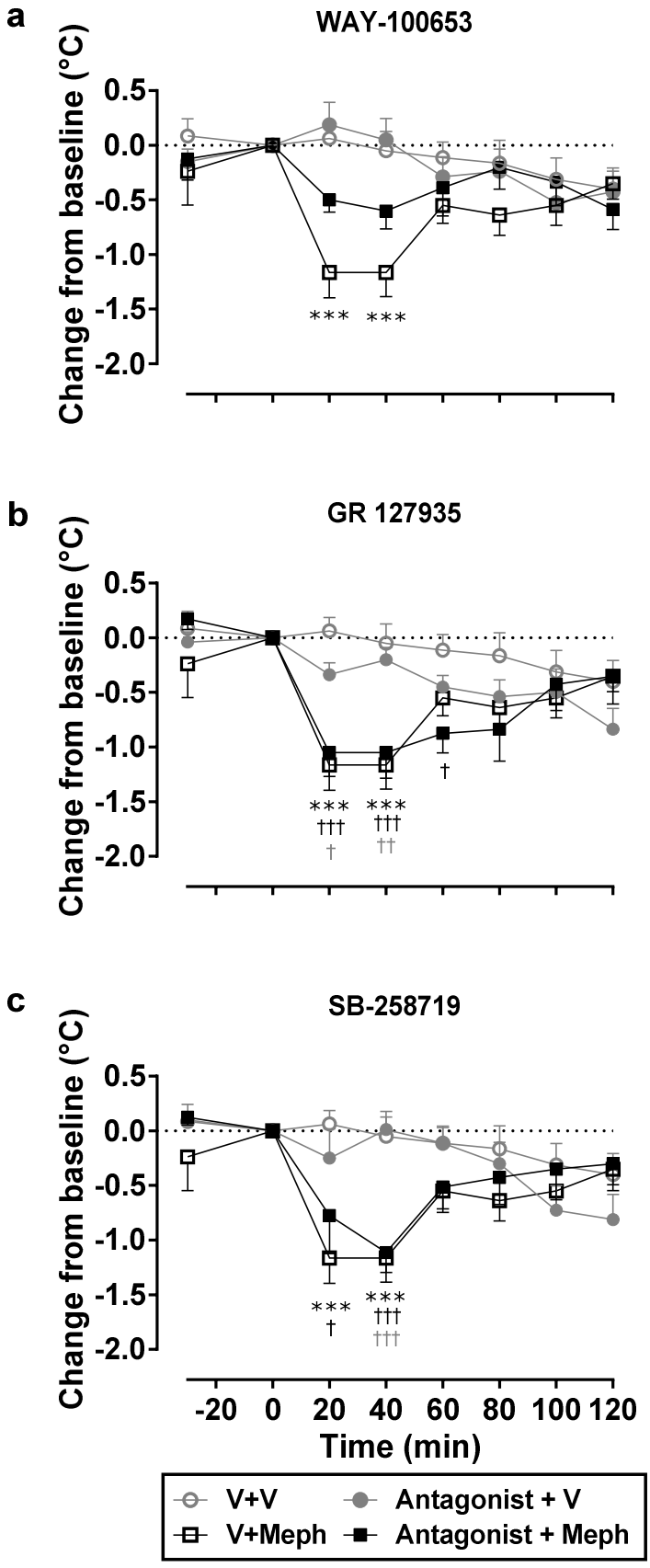

a
WAY-100653
Number of beam breaks
250
200
150
100
50
0
b
GR 127935
Number of beam breaks
c
SB-258719
Number of beam breaks
-30
-15
0
15
30
45
60
Time (min)
V+V
Antagonist + V
V+Meph
Antagonist + Meph

Figure 4


a
WAY-100653
Change from baseline (°C)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
***
***
b
GR 127935
Change from baseline (°C)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
***
†††
†
***
†††
††
†
c
SB-258719
Change from baseline (°C)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
***
†
***
†††
†††
-20
0
20
40
60
80
100
120
Time (min)
V+V
Antagonist + V
V+Meph
Antagonist + Meph