BJP British Journal of Pharmacology

DOI:10.1111/bph.12951
www.brjpharmacol.org

RESEARCH PAPER

Stereochemistry of mephedrone neuropharmacology: enantiomer-specific behavioural and neurochemical effects in rats

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Received
29 May 2014
Revised
11 September 2014
Accepted
20 September 2014

BACKGROUND AND PURPOSE

Synthetic cathinones, commonly referred to as 'bath salts', are a group of amphetamine-like drugs gaining popularity worldwide. 4-Methylmethcathinone (mephedrone, MEPH) is the most commonly abused synthetic cathinone in the UK, and exerts its effects by acting as a substrate-type releaser at monoamine transporters. Similar to other cathinone-related compounds, MEPH has a chiral centre and exists stably as two enantiomers: *R*-mephedrone (*R*-MEPH) and *S*-mephedrone (*S*-MEPH).

EXPERIMENTAL APPROACH

Here, we provide the first investigation into the neurochemical and behavioural effects of *R*-MEPH and *S*-MEPH. We analysed both enantiomers in rat brain synaptosome neurotransmitter release assays and also investigated their effects on locomotor activity (e.g. ambulatory activity and repetitive movements), behavioural sensitization and reward.

KEY RESULTS

Both enantiomers displayed similar potency as substrates (i.e. releasers) at dopamine transporters, but *R*-MEPH was much less potent than *S*-MEPH as a substrate at 5-HT transporters. Locomotor activity was evaluated in acute and repeated administration paradigms, with *R*-MEPH producing greater repetitive movements than *S*-MEPH across multiple doses. After repeated drug exposure, only *R*-MEPH produced sensitization of repetitive movements. *R*-MEPH produced a conditioned place preference whereas *S*-MEPH did not. Lastly, *R*-MEPH and *S*-MEPH produced biphasic profiles in an assay of intracranial self-stimulation (ICSS), but *R*-MEPH produced greater ICSS facilitation than *S*-MEPH.

CONCLUSIONS AND IMPLICATIONS

Our data are the first to demonstrate stereospecific effects of MEPH enantiomers and suggest that the predominant dopaminergic actions of *R*-MEPH (i.e. the lack of serotonergic actions) render this stereoisomer more stimulant-like when compared with *S*-MEPH. This hypothesis warrants further study.

Abbreviations

%MCR, % maximum control rate; CPP, conditioned place preference; DAT, dopamine transporter; ICSS, intracranial self-stimulation; MDA, methylenedioxyamphetamine; MDMA, 3,4,-methylenedioxymethamphetamine; MEPH, mephedrone (4-methylmethcathinone); MPP^+, 1-methyl-4-phenylpyridinium; *R*-MEPH, *R*-(d/(+))-mephedrone; SERT, 5-HT transporter; *S*-MEPH, *S*-(l/(-))-mephedrone

Tables of Links

TARGETS
GPCRs[a]
5-HT_2 receptor
Transporters[b]
DAT (dopamine transporter)
SERT (5-HT transporer)

LIGANDS	
5-HT	GBR12935
Amphetamine	MDMA
Citalopram	MPP+
Desipramine	Nomifensine
Dopamine	Reserpine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Synthetic cathinone abuse has increased at an alarming rate worldwide over the past few years. Often referred to as 'bath salts' or 'legal highs', synthetic cathinones are β-keto amphetamine compounds related to the parent compound cathinone (Carroll *et al.*, 2012). These compounds entered the recreational drug marketplace as substitutes for classical psychostimulants, such as methamphetamine and 3,4,-methylenedioxymethamphetamine (MDMA). Clandestine drug manufacturers popularized cathinones as 'legal high' alternatives to illegal psychostimulants with heavy Internet-based marketing, labelling synthetic cathinones as 'not for human consumption' (Deluca *et al*, 2009; Schifano *et al.*, 2011). These 'legal high' alternatives were also popularized due to an MDMA shortage following law enforcement crack-downs in many countries (Brunt *et al.*, 2011). Mephedrone (4-methylmethcathinone, MEPH) is the most commonly abused synthetic cathinone in the UK, and is widely abused worldwide. MEPH users report both cocaine-like stimulant properties and MDMA-like empathogenic properties (Deluca *et al*, 2009; Schifano *et al.*, 2011; Winstock *et al.*, 2011a,b). Legislation passed in the UK, US and worldwide has criminalized MEPH. Although some data suggest a reduction in MEPH use after criminalization, MEPH is still abused worldwide, often being sold under new 'legal high' brand titles (Brandt *et al.*, 2010; Winstock *et al.*, 2010; McElrath and O'Neill, 2011).

Similar to amphetamine and cathinone, MEPH has a chiral centre at its α-carbon and exists as two enantiomers, *R*-mephedrone (*R*-MEPH) and *S*-mephedrone (*S*-MEPH), which are sufficiently stable to racemization to allow for their independent *in vitro* and *in vivo* evaluation. All MEPH preclinical studies thus far have examined racemic MEPH effects. Racemic MEPH is thought to act pharmacologically by acting as a monoamine transporter substrate, thereby causing transporter-mediated extracellular release of dopamine (DA) and serotonin (5-HT) (Baumann *et al.*, 2012; López-Arnau *et al.*, 2012; Eshleman *et al.*, 2013; Opacka-Juffry *et al.*, 2014). This substrate action increases extracellular DA and 5-HT in the mesolimbic reward circuitry of rats (Kehr *et al.*, 2011; Baumann *et al.*, 2012). Racemic MEPH increases locomotor activity following acute exposure in rats and mice, and produces sensitization of repetitive movements following repeated exposure (López-Arnau *et al.*, 2012; Motbey *et al.*, 2012; Shortall *et al.*, 2012; Wright *et al.*, 2012; Gatch *et al.*, 2013; Gregg *et al.*, 2013a). In addition, racemic MEPH produces conditioned place preference (CPP), facilitates intracranial self-stimulation (ICSS) and is self-administered in rats (Hadlock *et al.*, 2011; Lisek *et al.*, 2012; Bonano *et al.*, 2013; Motbey *et al.*, 2013). These effects illustrate that racemic MEPH produces behavioural and neurochemical effects consistent with psychostimulant drugs that display high abuse liability.

Stereospecific effects of amphetamines and cathinone analogues structurally similar to MEPH have been studied. *S*-Cathinone is three times more potent than *R*-cathinone in causing *in vitro* DA release, while *S*-MDMA produces greater DA release in the striatum than *R*-MDMA (Kalix, 1986; Hiramatsu and Cho, 1990). *R*-methcathinone and *S*-methcathinone produce neurotoxicity in rat DA neurons but only *S*-methcathinone produces 5-HT neurotoxicity (Sparago *et al.*, 1996). *S*-methcathinone shows a threefold greater potency as a discriminative stimulus substituting for

cocaine compared with *R*-methcathinone in rats, and *S*-MDMA and racemic MDMA are more consistently reinforcing in self-administration than *R*-MDMA in rhesus monkeys (Glennon *et al.*, 1995; Wang and Woolverton, 2007). Given these stereospecific effects of methcathinone and similar analogues, the purpose of these studies was to characterize the neurochemical and behavioural effects of *R*-MEPH and *S*-MEPH in rats. The neurochemical profile of MEPH enantiomers was characterized using *in vitro* monoamine release assays targeting activity at dopamine transporters (DATs) and serotonin transporters (SERTs). Behaviourally, MEPH enantiomers were assessed for locomotor activity following acute and repeated administration. The rewarding properties of *R*-MEPH and *S*-MEPH were also evaluated using CPP and ICSS.

Methods

Animals and drugs

Male Sprague-Dawley rats (260–290 g; Harlan Laboratories, Indianapolis, IN, USA) were housed two per cage and maintained on a 12 h light/dark cycle for all ambulatory activity/repetitive movements and CPP experiments. The total number of animals used in these experiments was 301. Food and water were provided *ad libitum* except during testing. Animal use procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and Temple University Guidelines for the Care of Animals. For ICSS experiments, six adult male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing 342–366 g at the time of surgery were individually housed and maintained on a 12 h light/dark cycle. Rats were kept in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and food and water access *ad libitum* except during testing. Animal maintenance and research were in compliance with the NIH guidelines on Care and use of laboratory animals. All animal use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. All animal care and experimental procedures were in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Racemic MEPH (50:50 ratio of *R*-MEPH : *S*-MEPH), *R*-MEPH and *S*-MEPH were obtained from Fox Chase Chemical Diversity, Inc. Racemic MEPH was prepared using a literature method. *R*-MEPH (d-MEPH/(+)-MEPH) and *S*-MEPH (l-MEPH/(–)-MEPH) were prepared starting from natural amino acids in a way that stereochemistry is clearly known (see Supporting Information Appendix S1). *R*-MEPH and *S*-MEPH conformations are stable in the solid state, and undergo pH-dependent racemization in PBS buffer and rat plasma, where higher pH promotes greater deprotonation and racemization. Enantiomeric excess (ee), a measure of purity for each chiral enantiomer related to racemization, was used to determine the enantiomers' chiral purity in PBS solutions and rat plasma. Racemization results in twice as much of a loss of ee; for example, 5% racemization results in a 10% loss of ee. After 1.5 h at 37°C, a 5% racemization was observed for both the *R*-MEPH and *S*-MEPH in rat plasma, whereas in PBS buffer it was ~2% racemization. After 5 h at 37°C, the racemization increased to 25% in rat plasma and 6% in PBS buffer. Racemic MEPH, *R*-MEPH and *S*-MEPH were dissolved in physiological saline. Injections for all assays were administered i.p.

In vitro *transporter assays*

Male Sprague-Dawley rats (250–350 g) were killed by CO_2 narcosis and brains were processed to yield synaptosomes as previously described (Rothman *et al.*, 2001; 2003). Synaptosomes used in DAT release assays were prepared from rat striatum whereas synaptosomes in SERT release assays were prepared from whole brain minus striatum and cerebellum. For release assays, 9 nM [^{3}H]-MPP$^+$ was the radiolabelled substrate for DAT while 5 nM [^{3}H]-5-HT was the SERT substrate. MPP$^+$ was chosen as the radiolabelled substrate for DAT over [^{3}H]-dopamine for better stability and signal-to-noise ratio, as well as [^{3}H]-MPP$^+$ producing less diffusion out of the synaptosomes. All buffers used in the release assays contained 1 μM reserpine to block vesicular reuptake of substrates. Release assay selectivity was optimized for single transporters by including unlabelled blockers (100 nM desipramine and 100 nM citalopram for MPP$^+$ release, 100 nM nomifensine and 100 nM GBR12935 for 5-HT release) to prevent re-uptake of [^{3}H]-MPP$^+$ and [^{3}H]-5-HT by competing transporters. Synaptosomes were preloaded with radiolabelled substrated in Krebs-phosphate buffer for 1 h (steady state). Release assays were initiated by adding 850 μL of preloaded synaptosomes to 150 μL of test drug. Release was terminated by vacuum filtration through Whatman GF/B filters, and retained radioactivity was quantified by liquid scintillation counting.

Locomotor experiments: acute and repeated, intermittent dosing regiments

For all behavioural experimentation, rats were acclimatized in individual activity chambers for 60 min, during which activity was recorded. Activity post-drug injection was recorded for 90 min using a Digiscan DMicro (Accuscan, Inc., Columbus, OH, USA) (Lisek *et al.*, 2012; Gregg *et al.*, 2013a,b). Chambers consisted of transparent plastic boxes (45 cm × 20 cm × 20 cm) set inside metal frames equipped with 16 infrared light emitters and detectors. The number of photocell beam breaks was recorded by a computer interface and expressed as counts. Ambulatory activity was recorded as consecutive beam breaks resulting from horizontal movement. Non-ambulatory activity resulting in repetitive beam breaks was recorded as repetitive movements.

Two experiments were performed to assess ambulatory activity and repetitive movements following exposure to MEPH enantiomers. In the first experiment, rats (n = 8 per group) were administered a single dose of saline, *R*-MEPH or *S*-MEPH, and activity was measured. In the second experiment, rats (n = 8 per group) were given a variable-dose sensitization paradigm that produces sensitization of repetitive movements with racemic MEPH (Gregg *et al.*, 2013a). Saline, *R*-MEPH or *S*-MEPH was given for 7 days using the following doses: day 1 (15 mg·kg^{-1} *R*-MEPH/*S*-MEPH or saline), days 2–6 (30 mg·kg^{-1} *R*-MEPH/*S*-MEPH or saline), day 7 (15 mg·kg^{-1} *R*-MEPH/*S*-MEPH or saline). Following 10 days of drug abstinence, all groups were injected with 15 mg·kg^{-1} *R*-MEPH,

S-MEPH or saline, and activity was measured on the challenge day. Injections were conducted in home cages except for days during which activity was measured.

Conditioned place preference

CPP experiments (n = 7–8 per group) were conducted using a counterbalanced, biased design. CPP chambers (45 cm × 20 cm × 20 cm) consisted of two compartments separated by a removable door. Each compartment was environmentally distinguishable, with one compartment consisting of black walls and textured floor and the other consisting of vertical black and white stripes and a smooth floor. Each rat's CPP chamber preference was assessed during a 30 min preconditioning session during which rats were allowed access to both compartments and time spent in each compartment was recorded. A rat was considered to be in a compartment when all limbs entered the compartment. Time in each compartment was recorded manually by technicians blinded to individual animal treatments. The drug-paired compartment was designated as the non-preferred compartment during the preconditioning session. The 4 day conditioning phase began the day after preconditioning and at the same time of day for each rat. Rats received two conditioning sessions per day, one with an injection of *R*-MEPH or *S*-MEPH (for specific dosages, see Results section) and the other with a saline injection. Following drug or saline administration, rats were confined to the drug-paired/saline-paired compartment for 30 min. This confinement time was chosen to ensure optimal exposure levels of MEPH *in vivo* based on a 22 min *in vivo* half-life (Martínez-Clemente *et al*., 2013). Drug and saline injections were conducted 4 h apart and rats in the saline group received saline in both compartments. One day after the final conditioning session, rats were evaluated for place preference by allowing free exploration of both compartments in a drug-free state for 30 min, during which time spent on each side was recorded. Data are presented as a preference score, and calculated by taking the total time spent in the drug-paired compartment after conditioning minus the time in the drug-paired (non-preferred) compartment during the preconditioning session.

ICSS procedures

Before surgery, rats were anaesthetized with isoflurane (2.5–3% in oxygen; Webster Veterinary, Phoenix, AZ, USA) and then maintained with this anaesthetic during bipolar electrodes implantation (Plastics One, Roanoke, VA, USA). (The depth of anaesthesia and analgesia were assessed by monitoring responses to paw pressure and any changes in the rate of breathing.) The cathode was implanted into the left medial forebrain bundle at the level of the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral to midsagittal suture, 8.8 mm ventral to skull) using a stereotaxic device. Three screws were placed in the skull, and the anode was wrapped around the posterior screw to serve as a ground. The screws and electrode were secured to the skull with orthodontic resin. Ketoprofen (5 mg·kg^{-1}) was used for post-operative analgesia. Animals were allowed to recover for ≥7 days before ICSS training.

Experiments were conducted in sound-attenuating boxes containing acrylic test chambers (29.2 × 30.5 × 24.1 cm) equipped with a response lever (4.5 cm wide, 2.0 cm deep, 3 cm high), three stimulation lights, a 2 W house light and an ICSS stimulator (Med Associates, St. Albans, VT, USA). Electrodes were connected to the stimulator by a swivel commutator (Model SL2C; Plastics One). The stimulator, along with programming parameters and data acquisition, was controlled by Med-PC IV computer software (Med Associates).

Rats were trained under a fixed-ratio 1 schedule of electrical brain stimulation using a behavioural procedure identical to that previously described (Bonano *et al*., 2013). Each lever press resulted in delivery of a 0.5 s train of square wave cathodal pulses. During training, stimulation frequency was set at 126 Hz and intensity was adjusted for each rat to the lowest intensity that sustained a high reinforcement rate (>30 stimulations per minute). This intensity (100–160 μA) was held constant throughout the study and frequency manipulations were introduced. Sessions involving frequency manipulations consisted of three 10 min components. During each component, a descending series of 10 frequencies ranging from 158 to 56 Hz was presented. Each frequency trial began with a 10 s timeout during which responding had no scheduled consequences. Five non-contingent 'priming' stimulations were delivered during the last 5 s of the timeout to signal the stimulation frequency available during that trial. Non-contingent stimulation was followed by a 50 s 'response' period. Training continued until rats reliably responded at high rates for the first three to five frequency trials of each component over a period of ≥3 consecutive training days.

Test sessions lasted 90 min and consisted of three 10 min 'baseline' components, a 30 min timeout during which test compounds were administered and three 10 min 'test' components. *R*-MEPH (1.0–10 mg·kg^{-1}), *S*-MEPH (1.0–10 mg·kg^{-1}) or saline was administered 30 min before initiation of test components. Doses and pretreatment time were based on previous studies (Bonano *et al*., 2013). Test sessions were completed on Tuesdays and Fridays, and training sessions were conducted on all other weekdays. Testing and dose order with MEPH enantiomers was counterbalanced across rats for each enantiomer.

Data analysis

Statistical significance for all assays was set at $P < 0.05$. For synaptosome assays, EC_{50} values for stimulation of release were calculated using non-linear regression analysis. For the acute ambulatory activity/repetitive movements experiment, counts were summated into 5 min batches as a time course post-drug injection and analysed with a mixed three-way ANOVA (drug × dose × time) with time as the repeated factor. To evaluate individual MEPH enantiomers, mixed two-way ANOVAs (dose × time) were performed. Total repetitive movements and total ambulatory activity were analysed with two-way ANOVA and Bonferonni *post hoc* tests. For the repeated, intermittent sensitization paradigm, each dependent variable was analysed using a mixed three-way ANOVA (drug, previous drug exposure and time as factors) with time as the within-subjects factor and drug (*R*-MEPH and *S*-MEPH) and previous drug exposure (acute and repeated) as the between-subjects factor. To further investigate the repeated administration data interactions, mixed two-way ANOVAs (drug × time) were conducted with time as the repeated factor, and Bonferonni *post hoc* tests were employed to determine if sensitization was

observed. CPP experiments were analysed using one-way ANOVA and Bonferonni *post hoc* tests. For ICSS experiments, the primary dependent variable was reinforcement rate in stimulations min^{-1} during each frequency trial. To normalize these data, raw reinforcement rates from each trial in each rat were converted to % maximum control rate (%MCR), with MCR defined as the mean of the maximal rates observed during the second and third baseline components for any given rat in any given session. Thus, %MCR values were calculated as %MCR = (reinforcement rate during a frequency trial ÷ maximum control rate) × 100. For each experimental manipulation, data from all three test components were averaged within each rat and then across rats to yield mean test curves. Results were analysed by two-way ANOVA with Holm–Sidak *post hoc* tests using ICSS frequency and drug dose as factors. The total number of stimulations per component was calculated as the sum of stimulations delivered across all 10 frequency trials for each component. Test data were normalized to individual baseline data using the equation %baseline stimulations = (mean total stimulations per test component ÷ mean total stimulations per baseline component) × 100 averaged across rats. Peak changes produced in this summary measure by *R*-MEPH/*S*-MEPH were compared by *t*-test.

Results

R-*MEPH acts more selectively on dopamine transporters than* S-*MEPH*

Because it has been established that racemic MEPH functions as a substrate-type releaser at monoamine transporters, we compared the ability of *R*-MEPH, *S*-MEPH and racemic MEPH to evoke release by DAT and SERT *in vitro* (see Figure 1A and B). For dopamine release, EC_{50} values for *R*-MEPH, *S*-MEPH and racemic MEPH were 31.07, 74.23 and 54.31 nM respectively. For 5-HT release, EC_{50} values for *R*-MEPH, *S*-MEPH and racemic MEPH were 1.47 μM, 60.91 nM and 83.28 nM respectively. All of the drugs had similar potency at releasing [^{3}H]MPP+ via DAT. *R*-MEPH was a less potent releaser at SERT compared with *S*-MEPH and racemic MEPH and the racemate (i.e. higher EC_{50} value with *R*-MEPH than *S*-MEPH and racemate). The transporter selectivity of MEPH enantiomers was evaluated with DAT/SERT ratio comparisons. *R*-MEPH has a DAT/SERT ratio of 47 while *S*-MEPH has a DAT/SERT ratio of 0.8, demonstrating a 50-fold higher selectivity with *R*-MEPH for DAT.

Acute R-*MEPH produces greater repetitive movements than acute* S-*MEPH*

Activities produced by acute administrations of *R*-MEPH or *S*-MEPH are presented in Figure 2A–F. Repetitive movements (panel 2A–B) and ambulatory activity (panel 2C–D) are presented in summated counts in 5 min batches + SEM following *R*-MEPH or *S*-MEPH injection at 5, 10, 20 or 30 $mg \cdot kg^{-1}$. The three-way ANOVA comparing enantiomers was not significant for ambulatory activity [$F(1, 51) = 1.24$, $P = 0.63$] or repetitive movements [$F(1, 51) = 1.95$, $P = 0.52$]. For *R*-MEPH (panel 2A), significant effects of dose [$F(4, 76) = 135.5$, $P < 0.05$] and time [$F(19, 76) = 12.14$, $P < 0.05$] were identified for repetitive movements, and an interaction between dose and time [$F(19, 76) = 1.88$, $P < 0.001$] was observed. Significant effects of dose [$F(4, 76) = 112.3$, $P < 0.05$] and time [$F(19, 76) = 11.89$, $P < 0.05$] were observed for ambulatory activity with *R*-MEPH (panel 2C) and an interaction was observed [$F(19, 76) = 1.46$, $P = 0.008$]. For *S*-MEPH repetitive movements (panel 2B), significant effects of dose [$F(4, 76) = 78.86$, $P < 0.05$], time [$F(19, 76) = 11.40$, $P < 0.05$] and an interaction were observed [$F(19, 76) = 1.99$, $P < 0.001$]. For ambulatory activity with *S*-MEPH (panel 2D), significant effects of dose [$F(4, 76) = 70.42$, $P < 0.05$], time [$F(19, 76) = 17.88$, $P < 0.05$] and a significant interaction [$F(19, 76) = 2.18$, $P < 0.001$] were observed. For total repetitive movements (panel 2E), significant effects of treatment [$F(1, 3) = 30.36$, $P < 0.001$] and dose [$F(3, 3) = 8.97$, $P < 0.001$] were observed, with *post hoc* analysis identifying significantly greater total repetitive movements for *R*-MEPH over *S*-MEPH at 20 and 30 $mg \cdot kg^{-1}$ ($P < 0.001$). For total ambulatory activity (panel 2F), a significant effect of dose [$F(1, 3) = 7.49$, $P = 0.0003$] and treatment [$F(1, 3) = 4.43$, $P = 0.0398$] was also observed, with no differences observed between enantiomers at any dose in *post hoc* analyses.

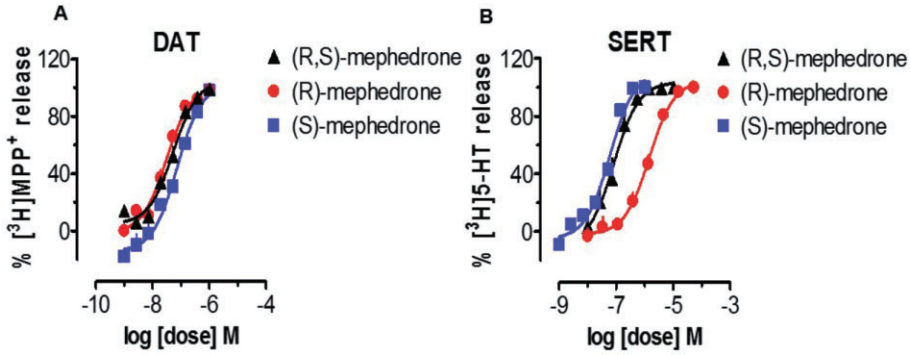

Figure 1

R-MEPH acts more selectively on dopamine transporters than *S*-MEPH. Drug concentration-response effects of *R*-MEPH, *S*-MEPH or racemic MEPH on facilitating monoamine release of [^{3}H]-MPP+ (A) and [^{3}H]-5-HT (B) *in vitro*. Concentration–response curves ($n = 3$ per dose) were constructed by incubating rat brain synaptosomes preloaded with tritiated substrate in increasing concentrations of each MEPH enantiomer with synaptosomes preloaded with tritiated substrate.

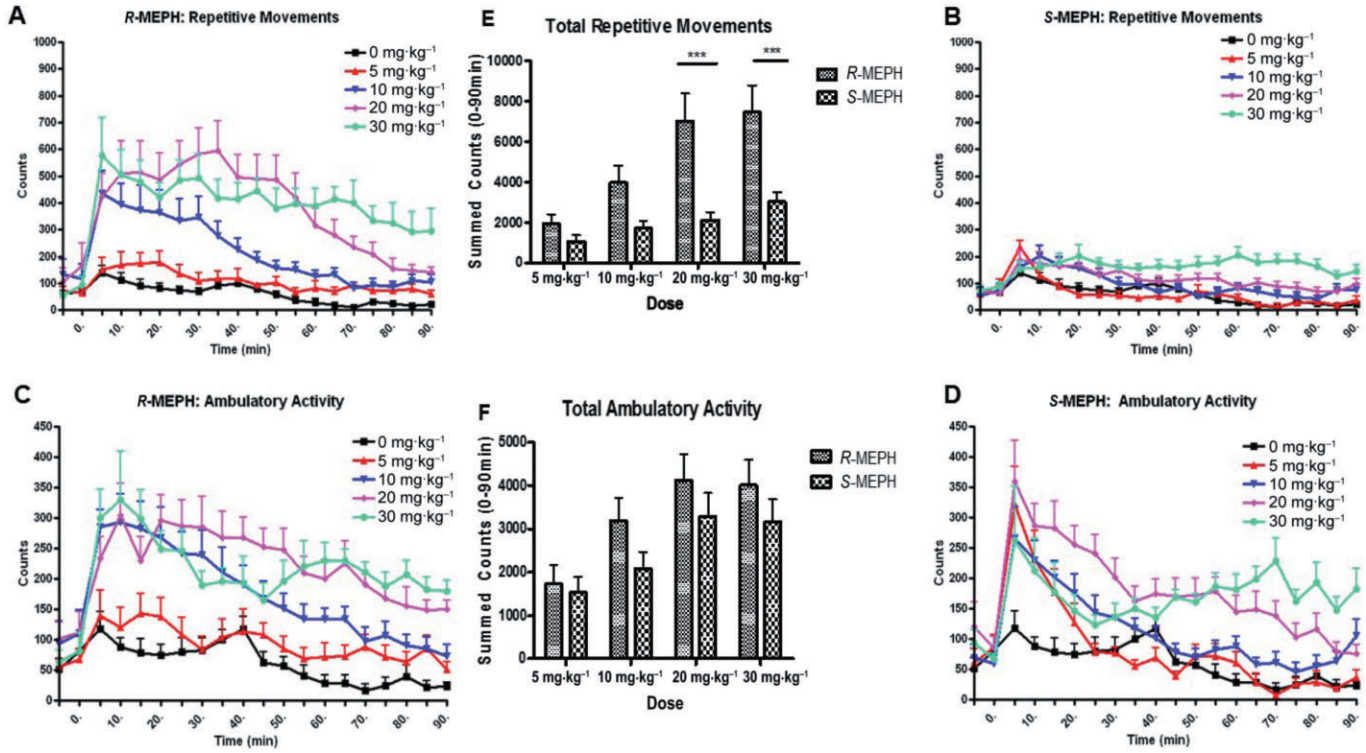

Figure 2

Acute *R*-MEPH produces greater repetitive movements than acute *S*-MEPH. Following drug injection, rats were monitored for 90 min for repetitive movements and ambulatory activity. Data are presented as a time course in 5 min batches (A–D) or as total counts over 90 min + SEM (E–F). For total repetitive movements and ambulatory activity analyses, ***$P < 0.001$ compared with saline control group.

R-*MEPH, but not* S-*MEPH, produces sensitization of repetitive movements*

Activities produced by repeated, intermittent *R*-MEPH/*S*-MEPH followed by 10 days of drug abstinence and a drug challenge are presented in Figure 3A and B. The three-way ANOVA found a significant effect for ambulatory activity [$F(17, 476) = 2.51$, $P < 0.001$] but not repetitive movements [$F(17, 476) = 0.94$, $P = 0.53$]. When analysed with two-way ANOVA to determine if sensitization was present, an overall effect was observed with treatment [$F(4, 76) = 145.65$, $P < 0.0001$] and time [$F(19, 84) = 19.52$, $P < 0.0001$] for repetitive movements (panel A). Increases in repetitive movements in rats given repeated, intermittent doses of *R*-MEPH compared with acute *R*-MEPH were observed at 40 min ($P < 0.01$) and 50 min ($P < 0.05$), while *S*-MEPH produced no sensitization of repetitive movements at any time point. For ambulatory activity (panel B), an overall effect was also observed with treatment [$F(4, 84) = 71.51$, $P < 0.0001$] and time [$F(19, 84) = 28.92$, $P < 0.001$]. No significant differences were observed between acute and repeated dosing paradigms for ambulatory activity for either *R*-MEPH or *S*-MEPH.

R-*MEPH, but not* S-*MEPH, produces dose-dependent place preference*

MEPH enantiomers were evaluated using our 4 day design for CPP. Data are represented as a preference score + SEM. Figure 4A presents a direct comparison of MEPH enantiomers and racemic MEPH at 20 mg·kg^{-1}; the first dose that *R*-MEPH and *S*-MEPH produced significantly different repetitive movements (Figure 2E). A significant overall effect was observed [$F(3, 26) = 5.347$, $P = 0.005$], with *post hoc* analysis showing *R*-MEPH, but not racemic MEPH, produced a greater preference score compared with both saline and *S*-MEPH ($P < 0.05$). *R*-MEPH did not produce a significantly greater preference than racemic MEPH. To further investigate MEPH enantiomer place preference, dose-response experiments were performed for each enantiomer at 5, 15 and 30 mg·kg^{-1} doses. Doses above 30 mg·kg^{-1} were not employed due to seizures observed at higher doses in pilot studies. *R*-MEPH (Figure 4B) at 30 mg·kg^{-1} produced a greater preference score than saline or 15 mg·kg^{-1} *R*-MEPH ($P < 0.05$). *S*-MEPH (Figure 4C) did not produce significant place preference compared with saline at any doses tested.

R-*MEPH produces greater ICSS facilitation than* S-*MEPH*

For the six rats in this study, the mean ± SEM baseline maximal control rate (MCR) was 58.1 ± 3.7 stimulations per trial, and the mean ± SEM baseline number of stimulations per component was 238.0 ± 22.1. Figure 5A–D shows the effects of *R*-MEPH and *S*-MEPH (1.0–10 mg·kg^{-1}) on ICSS. Panel 5A and C shows the effects on full frequency–rate curves. For *R*-MEPH (Figure 5A), two-way ANOVA indicated a main effect of frequency [$F(9, 45) = 33.45$, $P < 0.05$], but not

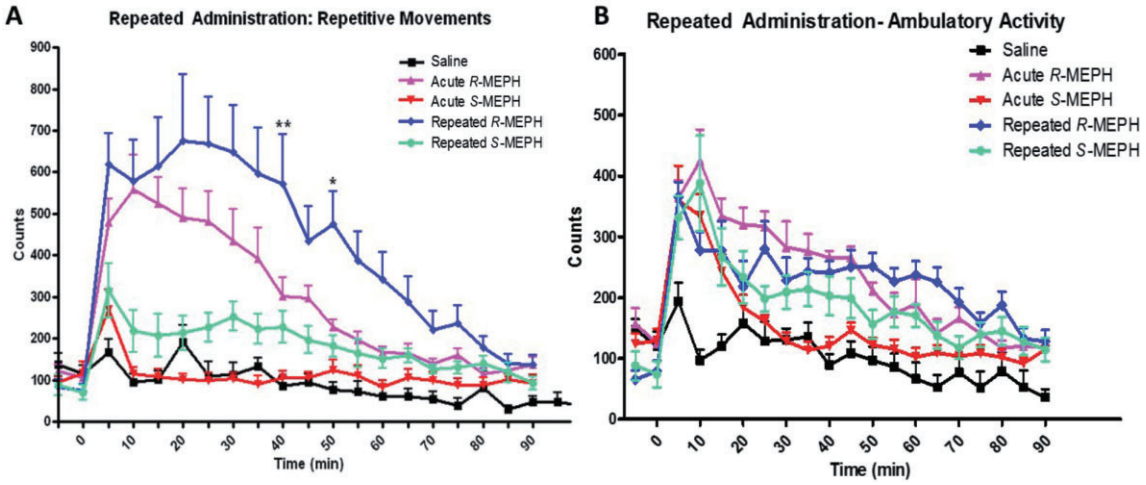

Figure 3

R-MEPH, but not *S*-MEPH, produces sensitization of repetitive movements. Rats (n = 8 per group) were given either saline or a repeated, variable-dose of *R*-MEPH or *S*-MEPH for 7 days, followed by a 10 day abstinence interval. After the abstinence interval, rats were challenged with either 15 mg·kg^{-1} *R*-MEPH or *S*-MEPH. Repetitive movements (A) and ambulatory activity (B) were monitored in 5 min bins and expressed as counts + SEM. *P < 0.05 and **P < 0.01 comparing rats given repeated *R*-MEPH to acute *R*-MEPH as determined by two-way ANOVA with Bonferonni *post hoc* tests.

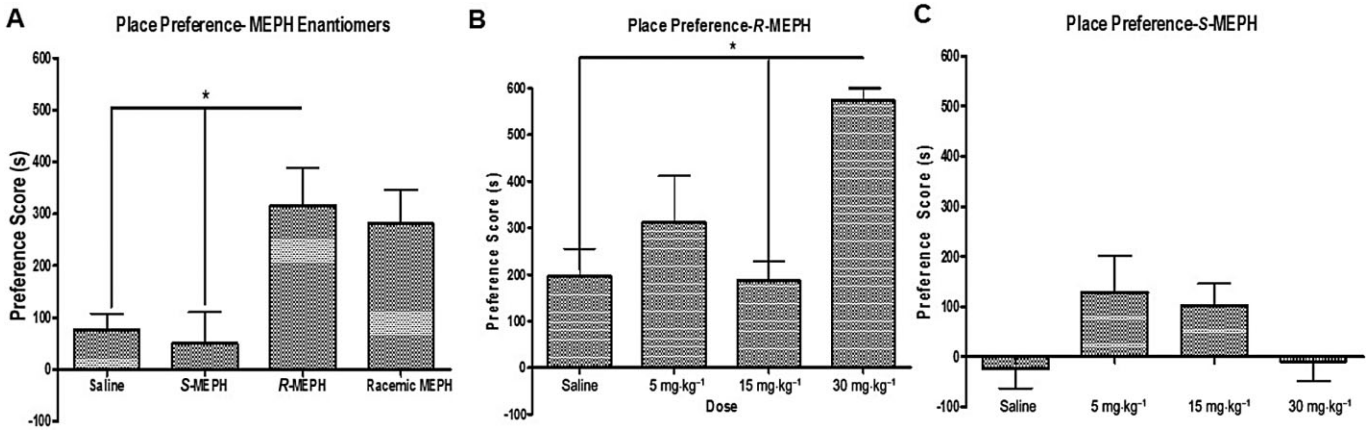

Figure 4

R-MEPH, but not *S*-MEPH, produces dose-dependent place preference. Rats (n = 7–8 per group) underwent a bias-design conditioned place preference assay, where drug was administered for 4 days in the non-preferred compartment, as determined by a 30 min pre-test in a drug-naïve state. Data are presented as a preference score (seconds on drug-paired side post-conditioning minus preconditioning)(s) + SEM. Each panel represents a cohort of animals with every panel having its own saline control group. Dose–response curves for *R*-MEPH (B) and *S*-MEPH (C), as well as a comparison with MEPH enantiomers and racemic MEPH at 20 mg·kg^{-1} (A), were performed. *P < 0.05 compared with saline control or indicated doses.

of dose [F(3, 15) = 1.536, P = 0.2463], and an interaction [F(27, 135) = 11.79, P < 0.05]. *R*-MEPH produced exclusive rate-increasing ICSS effects at 1.0 and 3.2 mg·kg^{-1} whereas 10 mg·kg^{-1} produced biphasic effects that included both increases in low ICSS rates maintained by low brain stimulation frequencies (1.75–1.95 log Hz) and decreases in high ICSS rates maintained by high frequencies (2.05–2.2 log Hz). For *S*-MEPH (Figure 5C), a two-way ANOVA revealed the main effects of frequency [F(9, 45) = 26.33, P < 0.05] and dose [F(3, 15) = 3.377, P < 0.05], and a frequency × dose interaction [F(27, 135) = 5.500, P < 0.05]. *S*-MEPH also produced exclusive rate-increasing effects at 1.0 and 3.2 mg·kg^{-1}, albeit to a lesser extent and across a narrower range of frequencies than *R*-MEPH. The higher dose of 10 mg·kg^{-1} *S*-MEPH produced exclusive depression of ICSS. Summary data show that peak facilitation of ICSS was produced by 3.2 mg·kg^{-1} of both *R*-MEPH (143 ± 10.3%) and *S*-MEPH (107 ± 14.4%) (Figure 5B and D). Maximum ICSS facilitation was greater for *R*-MEPH versus *S*-MEPH [t(5) = 3.54, P < 0.05].

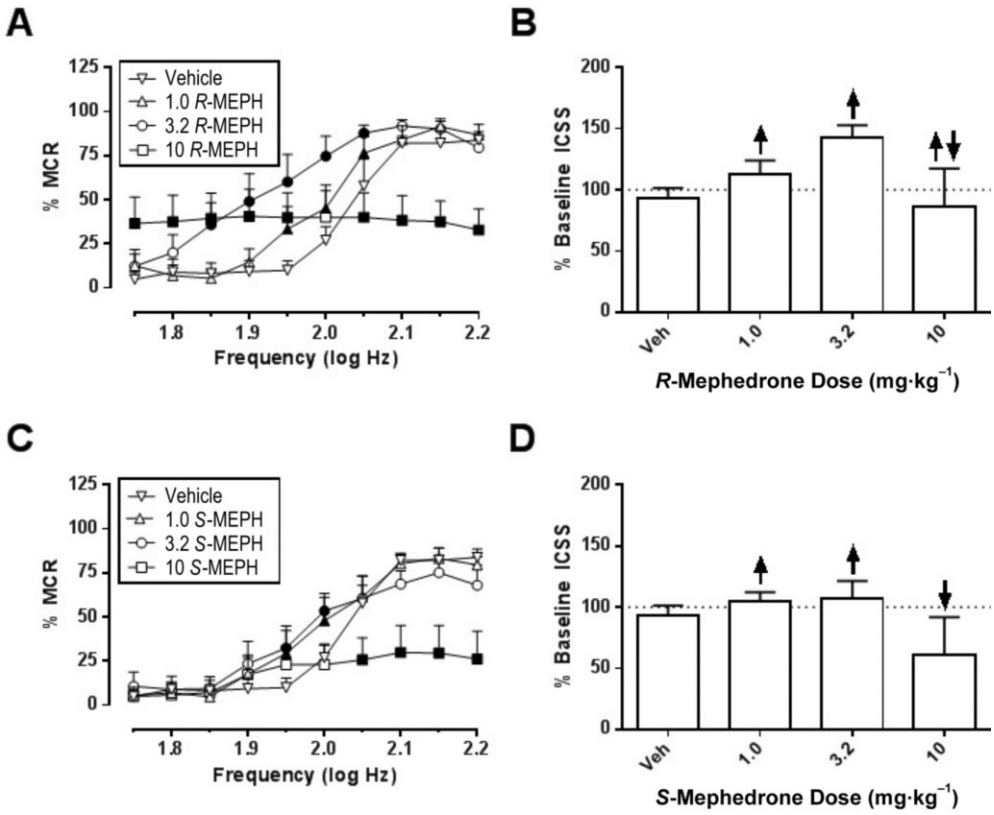

Figure 5

R-MEPH produces greater ICSS facilitation than *S*-MEPH. Left panels (A, C) show MEPH effects on full frequency–rate ICSS curves. Abscissae: frequency of electrical brain stimulation in log Hz. Ordinates: % maximum control reinforcement rate (%MCR). Drug doses indicated in keys are in units of mg·kg^{-1}. Solid symbols represent frequencies at which reinforcement rates were statistically different from vehicle rates as determined by two-way ANOVA followed by Holm–Sidak *post hoc* test ($P < 0.05$). Right panels (B, D) show MEPH effects on a summary measure of ICSS performance. Abscissae: drug dose in mg·kg^{-1}. Ordinates: % baseline number of stimulations per component (% baseline ICSS). Arrows indicate statistically significant increases (up arrows) and/or decreases (down arrows) in ICSS relative to vehicle at any frequency as determined from full frequency–rate curves. All data show mean ± SEM for six rats.

Discussion and conclusions

The major finding of this study is the substantial difference in the neuropharmacological profiles of *R*-MEPH and *S*-MEPH, with *S*-MEPH having a greater serotonergic profile and demonstrating mild locomotor activation and no rewarding properties among doses examined, and *R*-MEPH possessing more of a dopaminergic stimulant-like profile with both locomotor activation and reward. While *R*-MEPH and *S*-MEPH display similar effects on dopamine release, the *R*-stereoisomer is much weaker in its ability to release 5-HT. Using the DAT/SERT ratio, a metric that defines preference for drug-induced release effects at dopaminergic neurons over 5-HT neurons, *R*-MEPH displays a 50-fold greater preference for the dopaminergic system than *S*-MEPH.

Interestingly, for amphetamine, methamphetamine, methylenedioxyamphetamine and MDMA, the *S*-enantiomers produce greater synaptosomal dopamine release than the *R*-enantiomers (Arnold *et al.*, 1977; Johnson *et al.*, 1986; McKenna *et al.*, 1991; Kuczenski *et al.*, 1995). Few investigations comparing the effects of synthetic cathinone enantiomers on dopamine or 5-HT activity *in vitro* have been performed. To date, no direct comparisons have been performed to examine the effects of methcathinone enantiomers on dopamine or 5-HT release *in vitro*. Kalix (1986) found that *S*-cathinone was threefold more potent than *R*-cathinone in promoting dopamine release while Sparago *et al.* (1996) found that *R*-methcathinone was more potent than *S*-methcathinone in producing dopamine toxicity, but only *S*-methcathinone produced 5-HT neurotoxicity in rats. Although Sparago *et al.* only assessed neurotoxicity, their observed stereospecific effects may be due to greater neurotransmitter release causing that neurotoxicity. This could indicate a greater release of dopamine with *R*-methcathinone and greater release of 5-HT with *S*-methcathinone, as SERT substrate activity is directly related to long-term neurotoxic 5-HT depletions (Baumann *et al.*, 2014). As the only structural difference between MEPH and methcathinone is a *para* methyl ring substitution, it is possible that this methyl group contributes to the lack of stereospecificity at DAT observed with MEPH enantiomers versus methcathinone enantiomers, while having no effect on stereospecificity observed at SERT. Future studies will elaborate on the structure–activity relationship of MEPH enantiomers with monoamine transporters

through specific functional group manipulations, as well as using microdialysis to identify whether our *in vitro* findings correlate with *in vivo* changes in extracellular dopamine and 5-HT in brain reward circuits after drug administration.

The increase in ambulatory activity and repetitive movements following administration of MEPH suggests stereospecific effects as well. *R*-MEPH was much more efficacious in producing total repetitive movements than *S*-MEPH, while no difference in total ambulatory activity was observed with *R*-MEPH or *S*-MEPH. Increased repetitive movements versus ambulatory activity after MEPH enantiomer administration is similar to previously published results with racemic MEPH (Gregg *et al.*, 2013a). Additionally, in the variable-dosing schedule employed in our experiments, only *R*-MEPH produced sensitization of repetitive movements. While the observed sensitization of *R*-MEPH-induced repetitive movements is limited to specific 5 min intervals (40 and 50 min), this is similar to what is observed with racemic MEPH (Gregg *et al.*, 2013a). The finding that *R*-MEPH is more efficacious than *S*-MEPH in producing repetitive movements again differs from amphetamine and methamphetamine, where amphetamine and methamphetamine enantiomers produce no significantly different increases in stereotypy (Kuczenski *et al.*, 1995). No comparisons specifically analysing ambulatory activity or stereotypy/repetitive movements with cathinone or other synthetic cathinone enantiomers have been published as of this time. Coupled with the dopamine and 5-HT release assay data, MEPH enantiomers demonstrate a neurochemical and behavioural profile where *R*-MEPH displays more dopaminergic effects when compared with *S*-MEPH, contrary to observations with several similarly structured compounds.

CPP and ICSS are assays employed to investigate the rewarding effects of abused drugs, including psychostimulants (Tzschentke, 2007; Negus and Miller, 2014). Our previous studies have shown that racemic MEPH produces CPP at 30 mg·kg^{-1} (Lisek *et al.*, 2012). In the present CPP experiments, our first comparison examined *R*-MEPH, *S*-MEPH and racemic MEPH at 20 mg·kg^{-1}, the lowest dose at which significant differences in total repetitive movements were detected following acute administration. This initial experiment was followed by investigating each MEPH enantiomer across multiple doses. At a dose of 20 mg·kg^{-1}, *R*-MEPH produced greater preference scores than *S*-MEPH and saline. Dose-response experiments showed that *R*-MEPH also produces CPP at 30 mg·kg^{-1} whereas *S*-MEPH failed to produce CPP at any of the doses tested. Few studies have directly compared enantiomers of psychostimulants for their rewarding or reinforcing effects. Stereoisomers of amphetamine and MDMA evaluated in a rat CPP paradigm similar to the one employed here found *S*-amphetamine produced greater CPP than *R*-amphetamine while no differences between *R*-MDMA and *S*-MDMA were observed (Timár *et al.*, 1996; Meyer *et al.*, 2002). In progressive-ratio self-administration assays with rhesus monkeys, *S*-MDMA and racemic MDMA were consistently positive reinforcers while *R*-MDMA was a weak reinforcer, a finding that correlates to *S*-MDMA and racemic MDMA producing higher dopamine release than *R*-MDMA (Wang and Woolverton, 2007). While additional studies are needed to assess if synthetic cathinone reinforcing properties are stereospecific, our CPP data provide further support towards the *R*-enantiomer being the more rewarding enantiomer, while the opposite is observed with stimulants such as amphetamine and MDMA.

In ICSS experiments, racemic MEPH produced an abuse-related decrease in brain stimulation reward thresholds in mice and rats, although these threshold reductions were accompanied by reductions in maximal response rates (Robinson *et al.*, 2012; Bonano *et al.*, 2013). Previous studies have reported that effects of monoamine releasers on ICSS correlate with pharmacological selectivity to release dopamine versus 5-HT (Bauer *et al.*, 2013; Bonano *et al.*, 2013). Thus, dopamine-selective releasers (e.g. amphetamine or methcathinone) facilitate ICSS across a broad range of doses without reducing maximal rates, whereas 5-HT-selective releasers (e.g. fenfluramine) only depress ICSS. Relatively nonselective releasers, like racemic MDMA or MEPH, produce mixed effects on ICSS that typically include both increases in low ICSS rates maintained by low brain stimulation frequencies and decreases in high ICSS rates maintained by high stimulation frequencies. The main finding of this study was that *R*-MEPH produced greater ICSS facilitation than *S*-MEPH, consistent with its greater selectivity for DAT over SERT.

Studies have suggested that drugs producing preferential increases of 5-HT over dopamine produce lower rewarding effects in behavioural models like CPP and ICSS, and are less reinforcing in models like i.v. self-administration compared with drugs that act preferentially on dopamine. One of the mechanisms thought to be involved in these behavioural differences is 5-HT$_2$ receptor subtype activation that lowers extracellular levels of dopamine in the nucleus accumbens and striatum (De Deurwaerdère *et al.*, 2004; Navailles *et al.*, 2008; Huang *et al.*, 2011). Dopamine levels in the nucleus accumbens have been implicated in the rewarding effects of psychostimulants and are important in producing motivated behaviours (Roberts *et al.*, 1980; Ikemoto and Panksepp, 1999). This may explain why the dopamine-selective enantiomer *R*-MEPH produces a place preference while the less selective releaser *S*-MEPH produces no CPP. This may also explain why racemic MEPH produces weaker place preference than *R*-MEPH, perhaps through the release of 5-HT associated with *S*-MEPH diminishing the effects of the racemate. Self-administration studies would help determine if differences in reward properties of MEPH enantiomers observed in CPP assays correlate with similar stereospecificity of effects on reinforcing properties.

Understanding how stereochemistry influences the mechanism of action of MEPH is an important step in defining its neuropharmacological profile and identifying health risks posed by synthetic cathinones. The illicit manufacturing of synthetic cathinones like methcathinone and MEPH often involves synthesis procedures that result in one enantiomer being synthesized in larger quantities than the other, such as methcathinone primarily being synthesized as *S*-methcathinone (LeBelle *et al.*, 1995; Sparago *et al.*, 1996). To date, no investigations of the relative abundance of MEPH enantiomers in 'street' preparations of synthetic cathinone products have been conducted. Understanding these ratios of MEPH stereoisomers in the context of our studies may explain user reports of MEPH having both stimulant and empathogen-like effect. Additionally, understanding the ratios of these pharmacologically distinct enantiomers could

provide beneficial information to assist in designing strategies for targeted therapeutic interventions in MEPH abusers (Glennon, 2014), specifically based on which neurotransmitter systems are contributors to the abuse liability of illicit MEPH preparations.

Considering the observations reported here with *R*-MEPH and *S*-MEPH, and the prevalence of abuse of MEPH worldwide, it is important that these studies be done to better understand the mechanism of action of MEPH taken by abusers. We provide evidence here that MEPH enantiomers exhibit distinct stereospecific effects on neurochemistry and behaviour that are distinct when compared with drugs like amphetamine and MDMA. Future studies should characterize these stereospecific mechanisms in detail and provide valuable information on MEPH interactions with monoamine systems.

Acknowledgements

The authors would like to thank the National Institute on Drug Abuse for funding through grants DA025314, DA01342, DA032718, DA033930 and DA037649.

Author contributions

R. G. and S. M. contributed to data analysis, interpretation and writing the manuscript. M. B. and J. P. performed the *in vitro* release assays and data analysis on those assays. J. B. and S. N. performed the ICSS assays and data analysis on those assays. M. P. performed the three-way ANOVA analyses where applicable. A. R., G. S. and V. V. synthesized all racemic MEPH and MEPH enantiomers. R. G., C. T. and A. V. performed the acute ambulatory activity/repetitive movement assays, the behavioural sensitization assays and the CPP assays.

Conflict of interest

The authors report no conflicts of interest to disclose.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12951

Appendix S1 Chemical synthesis of racemic MEPH, 1 and 2.