Phase I metabolites of mephedrone display biological activity as substrates at monoamine transporters

Running short title: Bioactive metabolites of mephedrone

F P Mayer[1], L Wimmer[2], O Dillon-Carter[3], J S Partilla[3], N Burchardt[1], M D Mihovilovic[2], M H Baumann[3*], H H Sitte[1, 4*]

[1] *Medical University of Vienna, Center for Physiology and Pharmacology, Institute of Pharmacology, Vienna, Austria*

[2] *Institute of Applied Synthetic Chemistry, Vienna University of Technology, Vienna, Austria*

[3] *Designer Drug Research Unit (DDRU), Intramural Research Program (IRP), NIDA, NIH, Baltimore, MD, USA*

[4] *Center for Addiction Research and Science - AddRess, Medical University Vienna, Waehringerstrasse 13A, 1090 Vienna, Austria*

*denotes equal contribution

Correspondence

Harald S Sitte, MD, Institute of Pharmacology, Center for Physiology and Pharmacology, Medical University of Vienna, Währingerstrasse 13A, 1090 Vienna, Austria

E-mail: harald.sitte@meduniwien.ac.at

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.13547

BACKGROUND AND PURPOSE

4-Methyl-*N*-methylcathinone (mephedrone, MEPH) is a synthetic stimulant that acts as a substrate-type releaser at transporters for dopamine (DAT), norepinephrine (NET) and 5-HT (SERT). Upon systemic administration, MEPH is metabolized to several phase I compounds: the *N*-demethylated metabolite, 4-methylcathinone (NOR-MEPH); the ring-hydroxylated metabolite, 4-hydroxytolyl-mephedrone (4-OH-MEPH); and the reduced keto-metabolite, dihydromephedrone (DIHYDRO-MEPH).

EXPERIMENTAL APPROACH

We used *in vitro* assays to compare the effects of MEPH and synthetically prepared metabolites on transporter-mediated uptake and release in HEK293 cells expressing human monoamine transporters and in rat brain synaptosomes. *In vivo* microdialysis was employed to examine the effects of intravenous metabolite injection (1 and 3 mg kg^{-1}) on extracellular dopamine and 5-HT levels in rat nucleus accumbens.

KEY RESULTS

In cells expressing transporters, MEPH and its metabolites inhibited uptake, although DIHYDRO-MEPH was weak overall. In cells and synaptosomes, NOR-MEPH and 4-OH-MEPH served as transportable substrates, inducing release via monoamine transporters. When administered to rats, MEPH and NOR-MEPH produced elevations in extracellular dopamine and 5-HT, whereas 4-OH-MEPH did not. MEPH and NOR-MEPH, but not 4-OH-MEPH, induced locomotor activity.

CONCLUSIONS AND IMPLICATIONS

Our results demonstrate that phase I metabolites of MEPH are transporter substrates (*i.e.*, releasers) at DAT, NET and SERT, but DIHYDRO-MEPH is weak in this regard. When administered *in vivo*, NOR-MEPH increases extracellular dopamine and 5-HT in the brain whereas 4-OH-MEPH does not, suggesting the latter metabolite does not penetrate the blood-brain-barrier. Future studies should examine the pharmacokinetics of NOR-MEPH to determine its possible contribution to the *in vivo* effects produced by MEPH.

TARGETS	
Transporters[a]	**Enzymes**[b]
DAT, SLC6A3	CYP2D6
NET , SLC6A2	
SERT , SLC6A4	
VMAT2, SLC18A2	

LIGANDS	
Amphetamine	MDMA
Citalopram	MPP^+
Cocaine	Nomifensine
Desipramine	Norepinephrine
Dopamine	Serotonin,5-hydroxytryptamine, 5-HT
GBR12935	

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY ((Southan et al., 2016)), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a,b](Alexander et al., 2015a; Alexander et al., 2015b)).

Abbreviations

4-OH-MEPH, 4-hydroxytolylmephedrone; 5-HT, 5-hydroxytryptamine or serotonin; DAT, dopamine transporter; DIHYDRO-MEPH, dihydromephedrone; GBR12935, 1-(2-diphenylmethoxyethyl)-4-(3-phenylpropyl)piperazine dihydrochloride; MEPH, 4-methyl-*N*-methyl-cathinone or mephedrone; MPP^+, 1-methyl-4-phenylpyridinium; NET, norepinephrine transporter; NOR-MEPH, 4-methylcathinone; PDL, poly-D-lysine; SERT, 5-HT transporter

Introduction

During the past decade, a variety of man-made "designer drugs" or "new psychoactive substances" (NPS) have appeared in the recreational drug market as legal alternatives to more traditional drugs of abuse (Baumann, Solis, Watterson, Marusich, Fantegrossi & Wiley, 2014; Sitte & Freissmuth, 2015). Frequently, the chemical structures of NPS are based on known illicit substances and mimic their psychoactive effects, but subtle structural modifications to the drug molecules render them legal (Baumann & Volkow, 2016). In particular, a number of NPS have been marketed as replacements for illicit stimulants like cocaine and 3,4-

methylenedioxymethamphetamine (MDMA, "ecstasy") (Green, King, Shortall & Fone, 2014). One of the most popular synthetic stimulants is the cathinone analog, 4-methyl-*N*-methylcathinone (mephedrone, MEPH). MEPH first appeared in Israel as a "party drug" during the early 2000s, and its recreational use spread to Europe, Australia and other parts of the world (Kelly, 2011). In the United States, MEPH was a constituent of so-called "bath salts" products which became popular during 2010-2011 (Spiller, Ryan, Weston & Jansen, 2011). Low doses of MEPH produce typical stimulant effects in humans, like increased energy and mood elevation (Vardakou, Pistos & Spiliopoulou, 2011; Winstock, Mitcheson, Ramsey, Davies, Puchnarewicz & Marsden, 2011), while high doses or chronic use can produce life-threatening side-effects including tachycardia, hypertension, agitation and seizures (James et al., 2011; Wood, Greene & Dargan, 2011). Deaths from MEPH are rare but have been reported (Loi et al., 2015). In the interest of public health and safety, legislation was passed in many countries to ban the sale, possession and use of MEPH (Drug Enforcement Administration, 2011; Green, King, Shortall & Fone, 2014). Despite such bans, MEPH continues to be abused in European countries (Archer, Dargan, Lee, Hudson & Wood, 2014; Hondebrink, Nugteren-van Lonkhuyzen, Van Der Gouwe & Brunt, 2015) (http://www.emcdda.europa.eu/publications/edr/trends-developments/2014).

Similar to other stimulant drugs, MEPH exerts its effects by interacting with plasma membrane monoamine transporter proteins of the solute carrier 6 family (SLC6) (Baumann et al., 2012; Hadlock et al., 2011; Martinez-Clemente, Escubedo, Pubill & Camarasa, 2012), namely the dopamine transporter (DAT, SLC6A3), norepinephrine transporter (NET, SLC6A2) and serotonin (5-HT) transporter (SERT, SLC6A4). The normal role of monoamine transporters is to capture previously released neurotransmitter molecules from the extracellular space and move them back into the neuronal cytoplasm (*i.e*., uptake), thus terminating monoamine signaling (Kristensen et al., 2011; Reith et al., 2015). Drugs that interact with DAT, NET and SERT can be classified as either cocaine-like "blockers" or amphetamine-like "substrates" (Rothman & Baumann, 2003; Sitte & Freissmuth, 2015). Both types of compounds disrupt transporter function and produce elevations in extracellular monoamine concentrations, but their precise modes of action are different. On a molecular level, cocaine-like blockers act as non-transported inhibitors of monoamine transporters. Consequently, blockers prevent the transporter-mediated uptake of released neurotransmitters from the extracellular medium. In addition, cocaine is known to mobilize the intracellular reserve pool of DA and stimulate its exocytotic release (Venton et al., 2006)(Venton et al., 2006). In contrast, amphetamine-like compounds are transported substrates that not only act

as competitive uptake inhibitors but also trigger neurotransmitter efflux by a complex process involving reversal of transporter flux (Chen & Reith, 2004; Reith et al., 2015; Sitte & Freissmuth, 2015). Consequently, drugs that act as transporter substrates are often referred to as "releasers" as they induce a transporter-mediated efflux of neurotransmitters.

Studies using *in vitro* transporter assays in cells and rat brain synaptosomes have shown that MEPH acts as a non-selective substrate at DAT, NET and SERT, thereby leading to efflux of dopamine, norepinephrine and 5-HT (Baumann et al., 2012; Eshleman, Wolfrum, Hatfield, Johnson, Murphy & Janowsky, 2013; Simmler et al., 2013). Systemic administration of MEPH to rats increases the extracellular concentrations of dopamine and 5-HT in the brain, with the effects on 5-HT being somewhat greater in magnitude (Baumann et al., 2012; Kehr et al., 2011; Wright et al., 2012). Overall, the available preclinical data indicate that MEPH displays neurochemical effects that mimic MDMA, but MEPH has a number of physiological and toxicological properties that render it unique (Baumann et al., 2012; Miller et al., 2013; Shortall, Green, Swift, Fone & King, 2013). For example, high-dose administration of MEPH is less apt to produce robust hyperthermia and long-term depletions of brain tissue 5-HT (Baumann et al., 2012; den Hollander, Rozov, Linden, Uusi-Oukari, Ojanpera & Korpi, 2013; Motbey et al., 2012), effects that are well established for MDMA. Importantly, MEPH has greatly reduced potency at the vesicular monoamine transporter 2 (VMAT2, SLC18A2) when compared to MDMA and other ring-substituted amphetamines (Eshleman, Wolfrum, Hatfield, Johnson, Murphy & Janowsky, 2013; Pifl, Reither & Hornykiewicz, 2015), suggesting MEPH is less likely to disrupt intracellular stores of monoamine transmitters.

One possible explanation for the distinct effects of MEPH is that metabolites of the drug contribute to its *in vivo* profile of actions. Meyer et al. (2010) first reported that MEPH is metabolized by three main hepatic mechanisms (see Figure 1): 1) *N*-demethylation to form 4-methylcathinone or nor-mephedrone (NOR-MEPH); 2) hydroxylation of the 4-methyl ring-substitution to form 4-hydroxytolylmephedrone (4-OH-MEPH); and 3) reduction of the β-keto-oxygen group, which forms dihydromephedrone (DIHYDRO-MEPH) (Meyer, Wilhelm, Peters & Maurer, 2010). Pedersen and coworkers (2012) identified cytochrome P450 2D6 (CYP2D6) as the main enzyme responsible for the phase 1 metabolism of MEPH in humans, and detected NOR-MEPH, 4-OH-MEPH and DIHYDRO-MEPH in human urine specimens (Pedersen, Reitzel, Johansen & Linnet, 2013). As pointed out by Green et al. (Green, King, Shortall & Fone, 2014), no studies have examined the pharmacology of MEPH metabolites. Therefore in the present investigation, we used *in vitro* assays to compare the effects of

MEPH and its metabolites on transporter-mediated uptake and release in cells expressing human DAT, NET and SERT and in rat brain synaptosomes. Additionally, the *in vivo* neurochemical effects of systemically administered MEPH, NOR-MEPH or 4-OH-MEPH were examined using microdialysis in rat nucleus accumbens. Our data show that phase I metabolites of MEPH are substrates at monoamine transporters when assessed *in vitro*, but only NOR-MEPH displays substantial neurochemical actions *in vivo* which could contribute to the behavioral effects of systemically administered MEPH.

Methods

Animals and housing

Male Sprague-Dawley rats from Harlan Laboratories (Frederick, MD, USA) weighing 250-300 g at arrival were housed three per cage for two weeks prior to being used in experiments. The rats were housed under standard conditions (lights on from 0700-1900 h) with food and water available *ad libitum*. Rats were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and experiments were performed in accordance with the Institutional Care and Use Committee of the NIDA IRP. Rats used for brain tissue harvest to prepare synaptosomes were pair-housed, whereas those used in microdialysis experiments were single-housed post-operatively (see below).

Rat studies described herein were carried out in accordance with ARRIVE guidelines for reporting experiments involving animals. A total of 16 rats were used for *in vitro* synaptosome assays, and an additional 28 rats were used for *in vivo* microdialysis experiments.

Cell culture

The generation of human embryonic kidney 293 cells (HEK293 cells) stably expressing the human isoforms of DAT (hDAT) and NET (hNET) was carried out as described previously (Scholze, Norregaard, Singer, Freissmuth, Gether & Sitte, 2002). For SERT, the human isoform (hSERT) was cloned in frame with yellow fluorescent protein (YFP) (Schmid, Scholze, Kudlacek, Freissmuth, Singer & Sitte, 2001). The generation of a stable cell line was performed as described in Hilber and colleagues (Hilber et al., 2005). HEK293 cells were maintained in humidified atmosphere (5% CO_2, 37°C) in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % heat-inactivated fetal calf serum and penicillin

(100 IU 100 mL^{-1}) and streptomycin (100 µg 100 mL^{-1}). Selection pressure was maintained by adding geneticin (50 µg mL^{-1}) to the cell culture media.

Transporter uptake assays in HEK293 cells

Uptake experiments were conducted as described previously (Sitte, Hiptmair, Zwach, Pifl, Singer & Scholze, 2001) with minor modifications. In brief, HEK293 cells expressing hDAT, hNET or hSERT were seeded into poly-D-lysine (PDL) coated 96-well plates at a density of 40000 cells per well. The next day, DMEM was aspirated and replaced with Krebs HEPES buffer (KHB, 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgSO_4$, 5 mM D-glucose, pH adjusted to 7.3 with NaOH) (200 µL per well), and cells were pre-incubated with various concentrations of MEPH or its metabolites for 5 min (50 µL per well). Subsequently, 0.1 µM of [^{3}H]-5HT or 0.02 µM of [^{3}H]-MPP^+ were added and uptake was terminated after 1 (hSERT) or 3 min (hDAT, hNET) by washing the cells with 200 µL of ice-cold KHB. Cells were lysed with 1% sodium dodecyl sulphate (SDS) and tritium uptake was determined by scintillation counting. Nonspecific uptake was determined in presence of 10 µM paroxetine (hSERT) or 10 µM mazindol (hDAT and hNET).

Transporter release assays in HEK293 cells

Superfusion experiments were performed as described in Scholze and coworkers (Scholze, Norregaard, Singer, Freissmuth, Gether & Sitte, 2002). Briefly, HEK293 cells expressing the desired transporter were seeded at a density of 40000 cells per well onto poly-D-lysine-coated 5mm glass cover slips in 96-well plates 24 h prior to the experiment. Cells were preloaded with [^{3}H]-MPP^+ (0.1 µM, hDAT and hNET) or [^{3}H]-5HT (0.4 µM, hSERT) for 20 min at 37°C in a final volume of 100 µL per well. Subsequently, glass coverslips were transferred into small superfusion chambers (volume of 200 µL) and superfused with KHB at 25°C with a superfusion rate of 0.7 mL min^{-1} for 40 min to establish a stable basal efflux. After washout, the collection of two-minute fractions was initiated. After the first three basal fractions monensin (10 µM) or solvent were added for four fractions. Consequently, the cells were challenged with test-drugs (10 µM) for five fractions in presence or absence of monensin. Finally, the cells were lysed in 1% SDS to determine the total radioactivity. Radioactivity per fraction was assessed by a liquid scintillation counter and expressed as fractional release, *i.e.* the percentage of released ^{3}H in relation to total ^{3}H present at the beginning of the fraction (Sitte, Scholze, Schloss, Pifl & Singer, 2000). For analysis, release was expressed as area-under-the-curve (AUC). AUC was calculated for t=6 to 26 min and normalized to basal efflux, *i.e.* t=0 to 4 min.

Transporter release assays in rat brain synaptosomes

The ability of MEPH and its metabolites to evoke release via DAT, NET and SERT was determined in rat brain synaptosomes as previously described (Baumann et al., 2012). Rats were euthanized with CO_2, decapitated, and brains were rapidly removed and dissected on ice. Synaptosomes were prepared from striatum for DAT assays, whereas synaptosomes were prepared from whole brain minus striatum and cerebellum for the NET and SERT assays. [^{3}H]-MPP$^+$ (9 nM) was used as the radiolabeled substrate for DAT and NET, whereas [^{3}H]-5HT (5 nM) was used as the radiolabeled substrate for SERT. All buffers used in the release assays contained 1 µM reserpine to block vesicular uptake of substrates. The selectivity of assays was optimized for a single transporter by including unlabeled compounds (nomifensine and 1-(2-diphenylmethoxyethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (GBR12935) for SERT; GBR12935 and citalopram for NET; citalopram and desipramine for DAT) to prevent the uptake of [^{3}H]-MPP$^+$ or [^{3}H]-5HT by competing transporters. Synaptosomes were preloaded with radiolabeled substrate in Krebs-phosphate buffer (KPB) which consisted of 126 mM NaCl, 2.4 mM KCl, 0.5 mM KH_2PO_4, 1.1 mM $CaCl_2$, 0.83 mM $MgCl_2$, 0.5 mM Na_2SO_4, 11.1 mM glucose, 13.7 mM Na_2HPO_4, 1 mg mL^{-1} ascorbic acid, and 50 µM pargyline (pH=7.4) for 1 h (steady state). Assays were initiated by adding 850 µL of preloaded synaptosomes to 150 µL of test drug. Dose-response curves were generated using 8 different concentrations of MEPH, NOR-MEPH or 4-OH-MEPH. Assays were terminated by vacuum filtration, and retained radioactivity was quantified by liquid scintillation counting.

Microdialysis in Rat Nucleus Accumbens

In vivo microdialysis procedures were carried out as previously described with minor modifications (Baumann et al., 2012). Briefly, male rats anesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) received surgically-implanted jugular catheters and intracerebral guide cannulae aimed at the nucleus accumbens (AP +1.6 mm, ML -1.7 mm relative to bregma; -6.2 mm relative to dura)(Paxinos & Watson, 2007). After a 7-10 day recovery, each rat was placed into a chamber equipped with photobeams for the detection of motor parameters (TruScan, Harvard Apparatus, Holliston, MA, USA) and allowed to acclimate overnight. Food and water were available *ad libitum* during the acclimation period. On the following morning, catheters were attached to PE 50 extension tubes, and 0.5 x 2 mm

microdialysis probes (CMA/12, Harvard Apparatus, Holliston, MA, USA) were inserted into guide cannulae. Ringers' solution (150 mM NaCl , 2.8 mM KCl and 2.0 mM $CaCl_2$) was perfused through the probes at 0.6 µL min^{-1} for 3 h. To commence experiments, dialysate samples (20 µL) were collected at 20 min intervals, and drug or saline treatments were given after 3 baseline samples were obtained. Rats received two sequential intravenous (i.v.) injections of MEPH or its metabolites, with 1 mg kg^{-1} administered at time zero, followed by 3 mg kg^{-1} 60 min later. Saline was administered on the same schedule in a separate group of rats. Dialysate concentrations of dopamine and 5-HT were quantified using high-pressure liquid chromatography coupled to electrochemical detection (Baumann et al., 2012). Chromatographic data were exported to an Empower software system (Waters, Inc., Milford, MA, USA) for peak identification, integration and analysis.

Correct probe placements were assessed after the microdialysis experiments. Rats were euthanized by CO_2 narcosis then decapitated. Brains were quickly removed and immersion fixed in 10% paraformaldehyde for one week. Subsequently, brains were sectioned on a cryostat, and the location of each probe tip was verified by inspection of photographic images of the brain taken with a digital camera using the macro lens setting.

Analysis

Calculations were performed using Microsoft Excel® 2010 (Microsoft Corporation, Redmond, Washington, USA) and GraphPad Prism 5.0. (GraphPad Software Inc., La Jolla, California, USA). IC_{50} values for uptake inhibition and EC_{50} values for release were determined by nonlinear regression fits. Release data expressed as area-under-the-curve (AUC) were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Microdialysis and locomotor data were analyzed by two-way ANOVA (drug treatment x time) followed by Bonferroni's test. The effect of monensin treatment on basal efflux of tritiated substrate was analyzed with the Mann Whitney test. P values less than 0.05 (*i.e.*, $p<0.05$) were considered significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology(Curtis et al., 2015).

Materials

2-Methylamino-1-(*p*-tolyl)propan-1-one hydrochloride (MEPH, molecular weight (MW): 213.70), 2-amino-1-(*p*-tolyl)propan-1-one hydrochloride (NOR-MEPH, MW: 199.68) and 1-(4-(hydroxymethyl)phenyl)-2-(methylamino)propan-1-one hydrochloride (4-OH-MEPH,

MW: 229.70) were synthesized as racemic mixtures. In the case of 2-(methylamino)-1-(*p*-tolyl)propan-1-ol hydrochloride (DIHYDRO-MEPH, MW: 215.72) all four stereoisomers (syn-(1*R*,2*R*), syn-(1*S*,2*S*), anti-(1*R*,2*S*) and anti-(1*S*,2*R*)) were synthesized in their enantiopure form (99%ee) and tested as 1:1:1:1 mixture. Synthetic procedures and chemical characterization data are given in detail in the Supporting Information. Reagents used in the experiments for uptake inhibition and release in HEK293 cells were used as mentioned in previous work (Hofmaier et al., 2014). Plasmids encoding human SERT were a generous gift of Dr. Randy D. Blakely. For uptake and release experiments in HEK293-cells and rat brain synaptosomes, [^{3}H]-1-methyl-4-phenylpyridinium ([^{3}H]-MPP$^+$; 80-85 µCi mmol^{-1}) and [^{3}H]-5HT (28.3 µCi mmol^{-1}) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA) and Perkin Elmer (Boston, MA, USA), respectively. All other chemicals and cell culture supplies were from Sigma-Aldrich (St. Louis, MO, USA) with the exception of cell-culture dishes, which were obtained from Sarstedt (Nuembrecht, Germany).

Results

MEPH metabolites inhibit transporter-mediated uptake in HEK293 cells

We first tested the effects of MEPH and its metabolites on transporter-mediated uptake. Figure 2 shows that MEPH, NOR-MEPH, 4-OH-MEPH and DIHYDRO-MEPH were fully efficacious inhibitors of uptake in HEK293 cells stably expressing hDAT, hNET and hSERT. The potency of NOR-MEPH and 4-OH-MEPH to inhibit [^{3}H]-MPP$^+$ uptake via hDAT and hNET was comparable to MEPH, with IC_{50} values in the low micromolar range, from 0.7 to 6 µM. The IC_{50} values for DIHYDRO-MEPH to inhibit uptake via hDAT and hNET were much weaker (*i.e.*, 24 µM). Uptake inhibition experiments carried out with hSERT-expressing cells revealed that NOR-MEPH inhibited uptake in the low micromolar range with an IC_{50} value of 10.6 µM, whereas 4-OH- and DIHYDRO-MEPH were much less active with IC_{50} values exceeding 60 µM. The obtained IC_{50} values are shown in Table 1.

MEPH metabolites induce transporter-mediated release in HEK293 cells

Data from uptake inhibition assays cannot distinguish whether test drugs act as non-transported inhibitors or transportable substrates which evoke release (Baumann et al., 2013; Scholze, Zwach, Kattinger, Pifl, Singer & Sitte, 2000; Sitte, Scholze, Schloss, Pifl & Singer,

2000). Therefore, MEPH and its metabolites were tested in release assays to further explore their interaction with transporters. The release assays were performed with the same transporter-expressing HEK293 cell lines described above, and used a superfusion system (Sitte, Scholze, Schloss, Pifl & Singer, 2000). As described previously, efflux of preloaded [^{3}H]-MPP$^+$ or [^{3}H]-5HT was monitored in the presence or absence of monensin (10 µM) (Scholze, Zwach, Kattinger, Pifl, Singer & Sitte, 2000). Monensin acts as a selective H^+/Na^+ ionophore and dissipates the Na^+ gradient across cell membranes (Mollenhauer, Morre & Rowe, 1990): This compound increases the intracellular Na^+-concentration (Chen & Reith, 2004) and thus selectively enhances efflux triggered by transporter substrates. Importantly, only substrate-induced release will be enhanced by the application of monensin, while the effects of non-transported inhibitors will remain unchanged (Baumann et al., 2013; Sandtner et al., 2016; Scholze, Zwach, Kattinger, Pifl, Singer & Sitte, 2000). The superfusion assays performed here are a decisive tool to discriminate between inhibitors and substrates (Scholze, Zwach, Kattinger, Pifl, Singer & Sitte, 2000).

Time-course experiments with MEPH and its metabolites (10 µM) demonstrated that all of the agents evoked significant release of pre-loaded [^{3}H]-MPP$^+$ via hDAT and hNET, and release of pre-loaded [^{3}H]-5HT via hSERT. Figure 3A depicts a representative example of time-course effects for DAT-mediated release of [^{3}H]-MPP$^+$ induced by NOR-MEPH in the presence or absence of monensin. It is clear that monensin markedly enhanced the effects of NOR-MEPH on [^{3}H]-MPP$^+$ efflux. Additionally, monensin alone elicited a significant albeit modest increase in substrate release ($p<0.05$, Mann Whitney test), in agreement with our previous publications (Scholze et al., 2000). As a means to summarize the overall effect of test drugs on release, with and without monensin (10 µM), the data in Figure 3B-D are expressed as AUC for the 9 fractions collected after drug treatment. One-way ANOVA demonstrated that monensin significantly influenced the release of [^{3}H]-MPP$^+$ evoked by MEPH and its metabolites at DAT ($F_{7,91}=24.61$, $p<0.001$) and NET ($F_{7,85}=14.4$, $p<0.001$). Post-hoc analysis revealed that enhancement by monensin was significant for MEPH, NOR-MEPH and 4-OH-MEPH at hDAT and hNET, but not for DIHYDRO-MEPH. One-way ANOVA demonstrated that monensin significantly augmented the release of [^{3}H]-5HT ($F_{7,71}=31.68$, $p<0.001$) via hSERT, and this effect was significant for MEPH and all of its metabolites.

MEPH metabolites induce transporter-mediated release in synaptosomes

Next, we examined the effects of MEPH and its metabolites in rat brain synaptosomes to: i) analyze effects of test compounds in a native tissue preparation that contains plasma membrane transporters *in situ*; and ii) compare data from the human and rat transporters. MEPH, NOR- and 4-OH-MEPH were tested in release assays in rat brain synaptosomes, under conditions which were optimized for each transporter as described in Baumann and colleagues (Baumann et al., 2012). The dose-effect release data are depicted in Figure 4 and the calculated EC_{50} values are shown in Table 2. In comparison to the parent compound MEPH, NOR- and 4-OH-MEPH displayed only slightly reduced potencies as releasers of preloaded [^{3}H]-MPP^+ at DAT and NET, with EC_{50}s ranging from 0.05 µM to 0.22 µM (Figure 4 and Table 2). At SERT, NOR-MEPH induced release of pre-loaded [^{3}H]-5HT in a manner comparable to MEPH (EC_{50} = 0.2 µM), whereas a 10-fold rightward shift was detected for 4-OH-MEPH (EC_{50} = 2 µM).

NOR-MEPH, but not 4-OH-MEPH, affects neurochemistry and behavior in vivo

The findings from human and rat transporters agreed that MEPH, NOR-MEPH and 4-OH-MEPH were potent substrates at monoamine transporters. Thus, we sought to examine the neurochemical effects of these three compounds *in vivo*. Specifically, extracellular concentrations of dopamine and 5HT were assessed by microdialysis in the nucleus accumbens of freely-moving rats. As depicted in Figure 5, application of two-way ANOVA (drug treatment x time) demonstrated that drug treatments significantly influenced dialysate concentrations of dopamine ($F_{3,24}$=63.22, p<0.001) and 5-HT ($F_{3,24}$=83.83, p<0.001). Post-hoc tests revealed that MEPH increased dopamine after 1 mg kg^{-1}, whereas MEPH and NOR-MEPH both elevated dopamine after 3 mg kg^{-1}. 4-OH-MEPH had no significant impact on dopamine at either dose tested. MEPH and NOR-MEPH elevated dialysate concentrations of 5-HT in a nearly identical manner, with increases of 15-fold and 25-fold above baseline for the 1 and 3 mg kg^{-1} doses, respectively. Finally, drug treatments significantly affected motor behavior ($F_{3,24}$=36.05, p<0.001) such that MEPH and NOR-MEPH increased activity whereas 4-OH-MEPH did not. MEPH was more potent than NOR-MEPH as a locomotor stimulant, but both compounds significantly stimulated motor activity at the 3 mg kg^{-1} dose.

Discussion

The aim of the present study was to determine the pharmacological effects of phase I metabolites of MEPH and decipher their precise mode of action at monoamine transporters.

The synthetic cathinone MEPH has been shown to act as a non-selective, amphetamine-like substrate at monoamine transporters, thereby triggering release of dopamine, norepinephrine and 5-HT into the extracellular space (Baumann et al., 2012; Eshleman, Wolfrum, Hatfield, Johnson, Murphy & Janowsky, 2013; Simmler et al., 2013). The neurochemical effects of MEPH mimic those of MDMA (Baumann et al., 2012; Kehr et al., 2011; Wright et al., 2012) but MEPH has a number of distinct pharmacological effects when compared to MDMA and other ring-substituted amphetamines (reviewed by (Green, King, Shortall & Fone, 2014)). Many therapeutic and abused stimulant drugs - including diethylpropion, phendimetrazine and MDMA- are transformed by hepatic mechanisms into bioactive metabolites (Green, Mechan, Elliott, O'Shea & Colado, 2003; Rothman et al., 2002; Yu, Rothman, Dersch, Partilla & Rice, 2000). To examine whether metabolites of MEPH might be bioactive, we tested the known metabolites NOR-MEPH, 4-OH-MEPH and DIHYDRO-MEPH for their interactions with DAT, NET and SERT. It was found that all of the metabolites acted as substrate-type releasers, but NOR-MEPH and 4-OH-MEPH were much more potent than DIHYDRO-MEPH in this regard. Importantly, only NOR-MEPH influenced brain neurochemistry and behavior upon systemic administration.

The present *in vitro* data from HEK293 cells show that MEPH metabolites inhibit uptake in a concentration-dependent manner at all three plasma membrane monoamine transporters. NOR-MEPH and 4-OH-MEPH inhibited uptake at hDAT and hNET with potency comparable to MEPH, whereas DIHYDRO-MEPH was much weaker. Uptake inhibition assays can identify compounds that interact with monoamine transporters, but cannot discriminate whether such compounds act as inhibitors or substrates. Thus, we tested the effects of MEPH metabolites using release assays in HEK293 cells and rat brain synaptosomes. NOR-MEPH and 4-OH-MEPH evoked release of radiolabeled substrates from HEK293 cells stably expressing hDAT, hNET or hSERT. The releasing action of the drugs was augmented in the presence of monensin, an ionophore which dissipates Na^+ gradients across plasma membranes. The enhancement of release by monensin provides crucial mechanistic evidence that MEPH and its metabolites function mainly as transporter substrates, not merely as inhibitors, and thus are capable of inducing release of monoamines via their cognate transporters. Consistent with the data in HEK293 cells, NOR-MEPH and 4-OH-MEPH induced release of [^{3}H]-MPP^+ via DAT and NET, and release of [^{3}H]-5HT via SERT, in rat brain synaptosomes. Our findings with NOR-MEPH in synaptosomes agree with the recent findings of Hutsell et al. (Hutsell et al., 2015) who reported that stereoisomers

of NOR-MEPH (*i.e.*, stereoisomers of 4-methylcathinone) are non-selective transporter substrates that evoke neurotransmitter release from synaptosomes *in vitro.*

Previous investigations have revealed that the corresponding IC_{50} and EC_{50} values for a given drug to inhibit uptake or induce release may differ several-fold (Scholze, Zwach, Kattinger, Pifl, Singer & Sitte, 2000; Sitte, Hiptmair, Zwach, Pifl, Singer & Scholze, 2001). The apparent differences in potency that we observed here for inhibition of uptake (IC_{50} values in the µM range) versus stimulation of release (EC_{50} values in the nM range) might be attributed to different assay systems and methods used in our studies. For example, uptake assays in HEK293 cells use static incubation conditions, while release assays in HEK293 cells use dynamic perfusion conditions. Comparing results from release assays with HEK293 cells versus rat brain synaptosomes is even more problematic because the latter preparation consists of homogenized tissue that maximizes the surface area for drug-protein interactions. Additionally, HEK293 cells are non-neuronal in origin and do not possess all critical components of the plasma membrane protein machinery that are present in neurons *in vivo*. Despite the different assay systems and methods employed here, all of the findings agree that MEPH and its metabolites are substrates at monoamine transporters.

Even though the tested MEPH metabolites acted as transporter substrates *in vitro*, only NOR-MEPH significantly affected neurochemistry and behavior *in vivo*. The neurochemical profile of NOR-MEPH closely resembled that of MEPH at the doses tested in our study, but NOR-MEPH had weaker effects on extracellular dopamine and locomotion. Thus, it seems NOR-MEPH displays a more serotonergic profile of activity than the parent compound MEPH. The reduced locomotor response to NOR-MEPH as compared to MEPH is probably linked to blunted dopaminergic effects of the metabolite, because previous studies have shown that extracellular dopamine levels in the nucleus accumbens are tightly correlated with the extent of motor activation produced by stimulant drugs (Baumann, Clark, Woolverton, Wee, Blough & Rothman, 2011; Zolkowska et al., 2009)**.** Surprisingly, the systemic administration of 4-OH-MEPH had no significant effect on extracellular neurotransmitters in the brain or on behavior. Taken together with the *in vitro* findings, our *in vivo* data with 4-OH-MEPH suggest this metabolite may not penetrate through the blood-brain-barrier. The likelihood of substances to enter the brain is correlated with their size and lipid solubility (van Bree, de Boer, Danhof, Ginsel & Breimer, 1988; Waterhouse, 2003). Distribution coefficients calculated for MEPH and its metabolites indicate a clear-cut separation of lipohilic MEPH and NOR-MEPH on the one hand (logD7.4= 1.39 and 1.29, respectively) and hydrophilic 4-OH-MEPH on the other hand (logD7.4=0.14). As a

consequence, the increased hydrophilicity of 4-OH-MEPH, as compared to MEPH and NOR-MEPH, likely precludes the hydroxylated metabolite from entering the brain. We have noted a similar situation with the hydroxylated metabolites of MDMA, which are devoid of central activity when administered systemically to rats (Schindler, Thorndike, Blough, Tella, Goldberg & Baumann, 2014). Nevertheless, there is increasing evidence which points to the presence of various CYPs in brain tissue. Even though the expression levels of CYPs in the brain are low when compared to those in liver (Miksys & Tyndale, 2002), it is interesting to speculate that *in situ* metabolism of MEPH and formation of phase 1 metabolites in brain could impact on MEPH action *in vivo*. For instance, CYP2D6 has been detected in various regions of human brain, including substantia nigra and hippocampus (Siegle, Fritz, Eckhardt, Zanger & Eichelbaum, 2001). As a consequence, 4-OH-MEPH could be formed in close proximity to monoamine transporters and thereby contribute to the effects of MEPH. At present, there is no evidence that formation of metabolites in the central nervous system is of any pharmacological relevance. Interestingly, the dihydroxy metabolite of MDMA, 3,4-dihydroxymethamphetamine, displays potent stimulatory effects on heart rate and blood pressure upon systemic administration (Schindler, Thorndike, Blough, Tella, Goldberg & Baumann, 2014). Our results suggest that future investigations should examine the possible cardiovascular effects 4-OH-MEPH.

Furthermore, the most abundant mephedrone metabolite detected in forensic traffic cases was 4-OH-MEPH (Pedersen 2013). In two cases, however, the proportions of parental drug:metabolite blood plasma concentrations was 28:2 and 29:9 (all in µg/kg). In a number of cases, NOR-MEPH was detected up to five-fold higher as the internal standard amphetamine. While NOR-MEPH and DIHYDRO-MEPH were both detected in blood and urine samples, 4-OH-MEPH was mostly limited to urine, highlighting its hydrophilicity.

On the contrary, incubating MEPH with Sprague-Dawley rat liver hepatocytes for 30 minutes (Khreit et al., 2013) displayed the relative amounts of the MEPH metabolites: MEPH 59%, NOR-MEPH 23.5% and 4-OH-MEPH 6.16%. After 120 minutes Khreit et al. report the relative abundance as follows: MEPH 16%, NOR-MEPH 59% and 4-OH-MEPH 2.6%. However, further studies are needed to precisely determine the quantitative abundance of MEPH and its metabolites *in vivo* to estimate the contributions of the metabolites to the effects of MEPH.

The present data alone cannot clarify whether NOR-MEPH contributes to the psychoactive properties of systemically administered MEPH in animals or humans. Further studies are needed to determine the blood and brain concentrations of NOR-MEPH after

MEPH exposure. It is noteworthy that NOR-MEPH is the most abundant metabolite of MEPH identified in rats (Khreit, Grant, Zhang, Henderson, Watson & Sutcliffe, 2013; Martinez-Clemente, Lopez-Arnau, Carbo, Pubill, Camarasa & Escubedo, 2013) whereas 4-OH-MEPH is the major metabolite in humans (Pedersen, Reitzel, Johansen & Linnet, 2013; Pozo et al., 2015). Currently, no information is available on the pharmacokinetics and bioavailability of the metabolites after MEPH administration in either species. The collective results presented here demonstrate that phase I metabolites of MEPH are non-selective transporter substrates at DAT, NET and SERT, similar to the parent compound. However, only NOR-MEPH affects neurochemistry and behavior when administered peripherally, suggesting this metabolite could contribute significantly to the unique profile of psychoactive effects produced by MEPH. Further studies are warranted to examine this intriguing hypothesis.

Author contributions

F.P.M., O.D-C., J.S.P., L.W. and N.B. performed all experimental work, F.P.M., L.W., M.D.M., M.H.B. and H.H.S. designed the experiments, F.P.M., M.H.B. and H.H.S. wrote the manuscript and received significant input from all other authors.

Acknowledgements

We thank Dr. Oliver Kudlacek and Marion Holy for providing HEK293-cells stably expressing the human serotonin transporter and Dr. Lars Richter for calculating the distribution coefficients of mephedrone and its metabolites. The research was supported by Austrian Research Fund/FWF grants F3506 and W1232 (H.H.S.), the Intramural Research Program of the NIDA, NIH, grant DA000523-07 (M.H.B.), the FWF-DK MolTag, FWF W1232 (M.D.M.) and F.P.M. is a recipient of a DOC-fellowship of the Austrian Academy of Sciences.

Conflict of interest statement

HHS has received honoraria for lectures and consulting from AbbVie, Lundbeck, MSD, Ratiopharm, Roche, Sanofi-Aventis and Serumwerk Bernburg (past 5 years). All other authors declare no conflict of interest.

References

Alexander SP, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE*, et al.* (2015a). The Concise Guide to PHARMACOLOGY 2015/16: Enzymes. Br J Pharmacol 172**:** 6024-6109.

Alexander SP, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E*, et al.* (2015b). The Concise Guide to PHARMACOLOGY 2015/16: Transporters. Br J Pharmacol 172**:** 6110-6202.

Archer JR, Dargan PI, Lee HM, Hudson S, & Wood DM (2014). Trend analysis of anonymised pooled urine from portable street urinals in central London identifies variation in the use of novel psychoactive substances. Clin Toxicol (Phila) 52**:** 160-165.

Baumann MH, Ayestas MA, Jr., Partilla JS, Sink JR, Shulgin AT, Daley PF*, et al.* (2012). The designer methcathinone analogs, mephedrone and methylone, are substrates for monoamine transporters in brain tissue. Neuropsychopharmacology 37**:** 1192-1203.

Baumann MH, Clark RD, Woolverton WL, Wee S, Blough BE, & Rothman RB (2011). In vivo effects of amphetamine analogs reveal evidence for serotonergic inhibition of mesolimbic dopamine transmission in the rat. J Pharmacol Exp Ther 337**:** 218-225.

Baumann MH, Partilla JS, Lehner KR, Thorndike EB, Hoffman AF, Holy M*, et al.* (2013). Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive 'bath salts' products. Neuropsychopharmacology 38**:** 552-562.

Baumann MH, Solis E, Jr., Watterson LR, Marusich JA, Fantegrossi WE, & Wiley JL (2014). Baths salts, spice, and related designer drugs: the science behind the headlines. J Neurosci 34**:** 15150-15158.

Baumann MH, & Volkow ND (2016). Abuse of New Psychoactive Substances: Threats and Solutions. Neuropsychopharmacology 41**:** 663-665.

Chen N, & Reith ME (2004). Interaction between dopamine and its transporter: role of intracellular sodium ions and membrane potential. J Neurochem 89**:** 750-765.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA*, et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172**:** 3461-3471.

den Hollander B, Rozov S, Linden AM, Uusi-Oukari M, Ojanpera I, & Korpi ER (2013). Long-term cognitive and neurochemical effects of "bath salt" designer drugs methylone and mephedrone. Pharmacol Biochem Behav 103**:** 501-509.

Drug Enforcement Administration DoJ (2011). Schedules of controlled substances: temporary placement of three synthetic cathinones in Schedule I. Final Order. Fed Regist 76**:** 65371-65375.

Eshleman AJ, Wolfrum KM, Hatfield MG, Johnson RA, Murphy KV, & Janowsky A (2013). Substituted methcathinones differ in transporter and receptor interactions. Biochem Pharmacol 85**:** 1803-1815.

Green AR, King MV, Shortall SE, & Fone KC (2014). The preclinical pharmacology of mephedrone; not just MDMA by another name. Br J Pharmacol 171**:** 2251-2268.

Green AR, Mechan AO, Elliott JM, O'Shea E, & Colado MI (2003). The pharmacology and clinical pharmacology of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"). Pharmacol Rev 55**:** 463-508.

Hadlock GC, Webb KM, McFadden LM, Chu PW, Ellis JD, Allen SC*, et al.* (2011). 4-Methylmethcathinone (mephedrone): neuropharmacological effects of a designer stimulant of abuse. J Pharmacol Exp Ther 339**:** 530-536.

Hilber B, Scholze P, Dorostkar MM, Sandtner W, Holy M, Boehm S*, et al.* (2005). Serotonin-transporter mediated efflux: a pharmacological analysis of amphetamines and non-amphetamines. Neuropharmacology 49**:** 811-819.

Hofmaier T, Luf A, Seddik A, Stockner T, Holy M, Freissmuth M*, et al.* (2014). Aminorex, a metabolite of the cocaine adulterant levamisole, exerts amphetamine like actions at monoamine transporters. Neurochem Int 73**:** 32-41.

Hondebrink L, Nugteren-van Lonkhuyzen JJ, Van Der Gouwe D, & Brunt TM (2015). Monitoring new psychoactive substances (NPS) in The Netherlands: data from the drug market and the Poisons Information Centre. Drug Alcohol Depend 147**:** 109-115.

Hutsell BA, Baumann MH, Partilla JS, Banks ML, Vekariya R, Glennon RA*, et al.* (2015). Abuse-related neurochemical and behavioral effects of cathinone and 4-methylcathinone stereoisomers in rats. Eur Neuropsychopharmacol.

James D, Adams RD, Spears R, Cooper G, Lupton DJ, Thompson JP*, et al.* (2011). Clinical characteristics of mephedrone toxicity reported to the U.K. National Poisons Information Service. Emerg Med J 28**:** 686-689.

Kehr J, Ichinose F, Yoshitake S, Goiny M, Sievertsson T, Nyberg F*, et al.* (2011). Mephedrone, compared with MDMA (ecstasy) and amphetamine, rapidly increases both dopamine and 5-HT levels in nucleus accumbens of awake rats. Br J Pharmacol 164**:** 1949-1958.

Kelly JP (2011). Cathinone derivatives: a review of their chemistry, pharmacology and toxicology. Drug Test Anal 3**:** 439-453.

Khreit OI, Grant MH, Zhang T, Henderson C, Watson DG, & Sutcliffe OB (2013). Elucidation of the Phase I and Phase II metabolic pathways of (+/-)-4'-methylmethcathinone (4-MMC) and (+/-)-4'-(trifluoromethyl)methcathinone (4-TFMMC) in rat liver hepatocytes using LC-MS and LC-MS(2). J Pharm Biomed Anal 72**:** 177-185.

Kristensen AS, Andersen J, Jorgensen TN, Sorensen L, Eriksen J, Loland CJ*, et al.* (2011). SLC6 neurotransmitter transporters: structure, function, and regulation. Pharmacol Rev 63**:** 585-640.

Loi B, Corkery JM, Claridge H, Goodair C, Chiappini S, Gimeno Clemente C*, et al.* (2015). Deaths of individuals aged 16-24 years in the UK after using mephedrone. Hum Psychopharmacol 30**:** 225-232.

Martinez-Clemente J, Escubedo E, Pubill D, & Camarasa J (2012). Interaction of mephedrone with dopamine and serotonin targets in rats. Eur Neuropsychopharmacol 22**:** 231-236.

Martinez-Clemente J, Lopez-Arnau R, Carbo M, Pubill D, Camarasa J, & Escubedo E (2013). Mephedrone pharmacokinetics after intravenous and oral administration in rats: relation to pharmacodynamics. Psychopharmacology (Berl) 229**:** 295-306.

Meyer MR, Wilhelm J, Peters FT, & Maurer HH (2010). Beta-keto amphetamines: studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography-mass spectrometry. Anal Bioanal Chem 397**:** 1225-1233.

Miksys SL, & Tyndale RF (2002). Drug-metabolizing cytochrome P450s in the brain. J Psychiatry Neurosci 27**:** 406-415.

Miller ML, Creehan KM, Angrish D, Barlow DJ, Houseknecht KL, Dickerson TJ*, et al.* (2013). Changes in ambient temperature differentially alter the thermoregulatory, cardiac and locomotor stimulant effects of 4-methylmethcathinone (mephedrone). Drug Alcohol Depend 127**:** 248-253.

Mollenhauer HH, Morre DJ, & Rowe LD (1990). Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. Biochim Biophys Acta 1031**:** 225-246.

Motbey CP, Karanges E, Li KM, Wilkinson S, Winstock AR, Ramsay J*, et al.* (2012). Mephedrone in adolescent rats: residual memory impairment and acute but not lasting 5-HT depletion. PLoS One 7**:** e45473.

Paxinos G, & Watson C (2007) *The rat brain in stereotaxic coordinates*. 6th edn. Elsevier: Amsterdam ; Boston.

Pedersen AJ, Reitzel LA, Johansen SS, & Linnet K (2013). In vitro metabolism studies on mephedrone and analysis of forensic cases. Drug Test Anal 5**:** 430-438.

Pifl C, Reither H, & Hornykiewicz O (2015). The profile of mephedrone on human monoamine transporters differs from 3,4-methylenedioxymethamphetamine primarily by lower potency at the vesicular monoamine transporter. Eur J Pharmacol 755**:** 119-126.

Pozo OJ, Ibanez M, Sancho JV, Lahoz-Beneytez J, Farre M, Papaseit E*, et al.* (2015). Mass spectrometric evaluation of mephedrone in vivo human metabolism: identification of phase I and phase II metabolites, including a novel succinyl conjugate. Drug Metab Dispos 43**:** 248-257.

Reith ME, Blough BE, Hong WC, Jones KT, Schmitt KC, Baumann MH*, et al.* (2015). Behavioral, biological, and chemical perspectives on atypical agents targeting the dopamine transporter. Drug Alcohol Depend 147**:** 1-19.

Rothman RB, & Baumann MH (2003). Monoamine transporters and psychostimulant drugs. Eur J Pharmacol 479**:** 23-40.

Rothman RB, Katsnelson M, Vu N, Partilla JS, Dersch CM, Blough BE*, et al.* (2002). Interaction of the anorectic medication, phendimetrazine, and its metabolites with monoamine transporters in rat brain. Eur J Pharmacol 447**:** 51-57.

Sandtner W, Stockner T, Hasenhuetl PS, Partilla JS, Seddik A, Zhang YW*, et al.* (2016). Binding Mode Selection Determines the Action of Ecstasy Homologs at Monoamine Transporters. Mol Pharmacol 89**:** 165-175.

Schindler CW, Thorndike EB, Blough BE, Tella SR, Goldberg SR, & Baumann MH (2014). Effects of 3,4-methylenedioxymethamphetamine (MDMA) and its main metabolites on cardiovascular function in conscious rats. Br J Pharmacol 171**:** 83-91.

Schmid JA, Scholze P, Kudlacek O, Freissmuth M, Singer EA, & Sitte HH (2001). Oligomerization of the human serotonin transporter and of the rat GABA transporter 1 visualized by fluorescence resonance energy transfer microscopy in living cells. J Biol Chem 276**:** 3805-3810.

Scholze P, Norregaard L, Singer EA, Freissmuth M, Gether U, & Sitte HH (2002). The role of zinc ions in reverse transport mediated by monoamine transporters. J Biol Chem 277**:** 21505-21513.

Scholze P, Zwach J, Kattinger A, Pifl C, Singer EA, & Sitte HH (2000). Transporter-mediated release: a superfusion study on human embryonic kidney cells stably expressing the human serotonin transporter. J Pharmacol Exp Ther 293**:** 870-878.

Shortall SE, Green AR, Swift KM, Fone KC, & King MV (2013). Differential effects of cathinone compounds and MDMA on body temperature in the rat, and pharmacological characterization of mephedrone-induced hypothermia. Br J Pharmacol 168**:** 966-977.

Siegle I, Fritz P, Eckhardt K, Zanger UM, & Eichelbaum M (2001). Cellular localization and regional distribution of CYP2D6 mRNA and protein expression in human brain. Pharmacogenetics 11**:** 237-245.

Simmler LD, Buser TA, Donzelli M, Schramm Y, Dieu LH, Huwyler J*, et al.* (2013). Pharmacological characterization of designer cathinones in vitro. Br J Pharmacol 168**:** 458-470.

Sitte HH, & Freissmuth M (2015). Amphetamines, new psychoactive drugs and the monoamine transporter cycle. Trends Pharmacol Sci 36**:** 41-50.

Sitte HH, Hiptmair B, Zwach J, Pifl C, Singer EA, & Scholze P (2001). Quantitative analysis of inward and outward transport rates in cells stably expressing the cloned human serotonin transporter: inconsistencies with the hypothesis of facilitated exchange diffusion. Mol Pharmacol 59**:** 1129-1137.

Sitte HH, Scholze P, Schloss P, Pifl C, & Singer EA (2000). Characterization of carrier-mediated efflux in human embryonic kidney 293 cells stably expressing the rat serotonin transporter: a superfusion study. J Neurochem 74**:** 1317-1324.

Southan C, Sharman JL, Benson HE, Faccenda E, Pawson AJ, Alexander SP*, et al.* (2016). The IUPHAR/BPS Guide to PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. Nucleic Acids Res 44**:** D1054-1068.

Spiller HA, Ryan ML, Weston RG, & Jansen J (2011). Clinical experience with and analytical confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. Clin Toxicol (Phila) 49**:** 499-505.

van Bree JB, de Boer AG, Danhof M, Ginsel LA, & Breimer DD (1988). Characterization of an "in vitro" blood-brain barrier: effects of molecular size and lipophilicity on cerebrovascular endothelial transport rates of drugs. J Pharmacol Exp Ther 247**:** 1233-1239.

Vardakou I, Pistos C, & Spiliopoulou C (2011). Drugs for youth via Internet and the example of mephedrone. Toxicol Lett 201**:** 191-195.

Venton BJ, Seipel AT, Phillips PE, Wetsel WC, Gitler D, Greengard P*, et al.* (2006). Cocaine increases dopamine release by mobilization of a synapsin-dependent reserve pool. J Neurosci 26**:** 3206-3209.

Waterhouse RN (2003). Determination of lipophilicity and its use as a predictor of blood-brain barrier penetration of molecular imaging agents. Mol Imaging Biol 5**:** 376-389.

Winstock A, Mitcheson L, Ramsey J, Davies S, Puchnarewicz M, & Marsden J (2011). Mephedrone: use, subjective effects and health risks. Addiction 106**:** 1991-1996.

Wood DM, Greene SL, & Dargan PI (2011). Clinical pattern of toxicity associated with the novel synthetic cathinone mephedrone. Emerg Med J 28**:** 280-282.

Wright MJ, Jr., Angrish D, Aarde SM, Barlow DJ, Buczynski MW, Creehan KM*, et al.* (2012). Effect of ambient temperature on the thermoregulatory and locomotor stimulant effects of 4-methylmethcathinone in Wistar and Sprague-Dawley rats. PLoS One 7**:** e44652.

Yu H, Rothman RB, Dersch CM, Partilla JS, & Rice KC (2000). Uptake and release effects of diethylpropion and its metabolites with biogenic amine transporters. Bioorg Med Chem 8**:** 2689-2692.

Zolkowska D, Jain R, Rothman RB, Partilla JS, Roth BL, Setola V*, et al.* (2009). Evidence for the involvement of dopamine transporters in behavioral stimulant effects of modafinil. J Pharmacol Exp Ther 329**:** 738-746.

EMCDDA, Lisbon, European Drug Report 2014: Trends and developments [Online] Available from http://www.emcdda.europa.eu/publications/edr/trends-developments/2014 [Accessed: 30th May 2016]

Figures and Figure Legends:

Five Figures

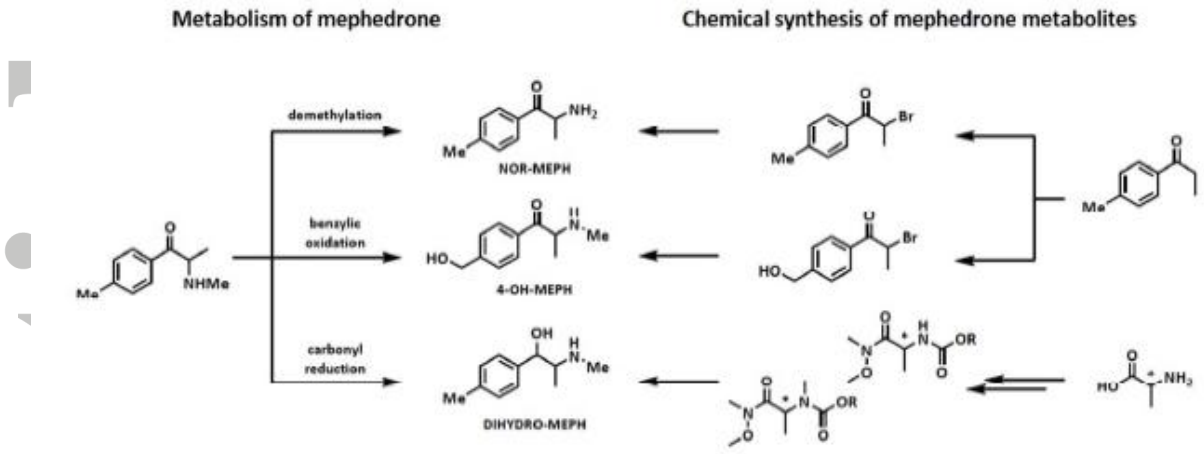

Figure 1. Proposed pathways for metabolism of mephedrone (MEPH) to its phase I metabolites. (1) *N*-demethylation forms 4-methylcathinone (NOR-MEPH); (2) *para* hydroxylation forms 4-hydroxytolylmephedrone (4-OH-MEPH); (3) ß-keto reduction forms dihydromephedrone (DIHYDRO-MEPH). Chemical synthesis started from non-chiral precursors for the generation of racemic NOR-MEPH and 4-OH-MEPH and from chiral precursors for DIHYDRO-MEPH (racemic diastereomers obtained by mixing of enantiomers)

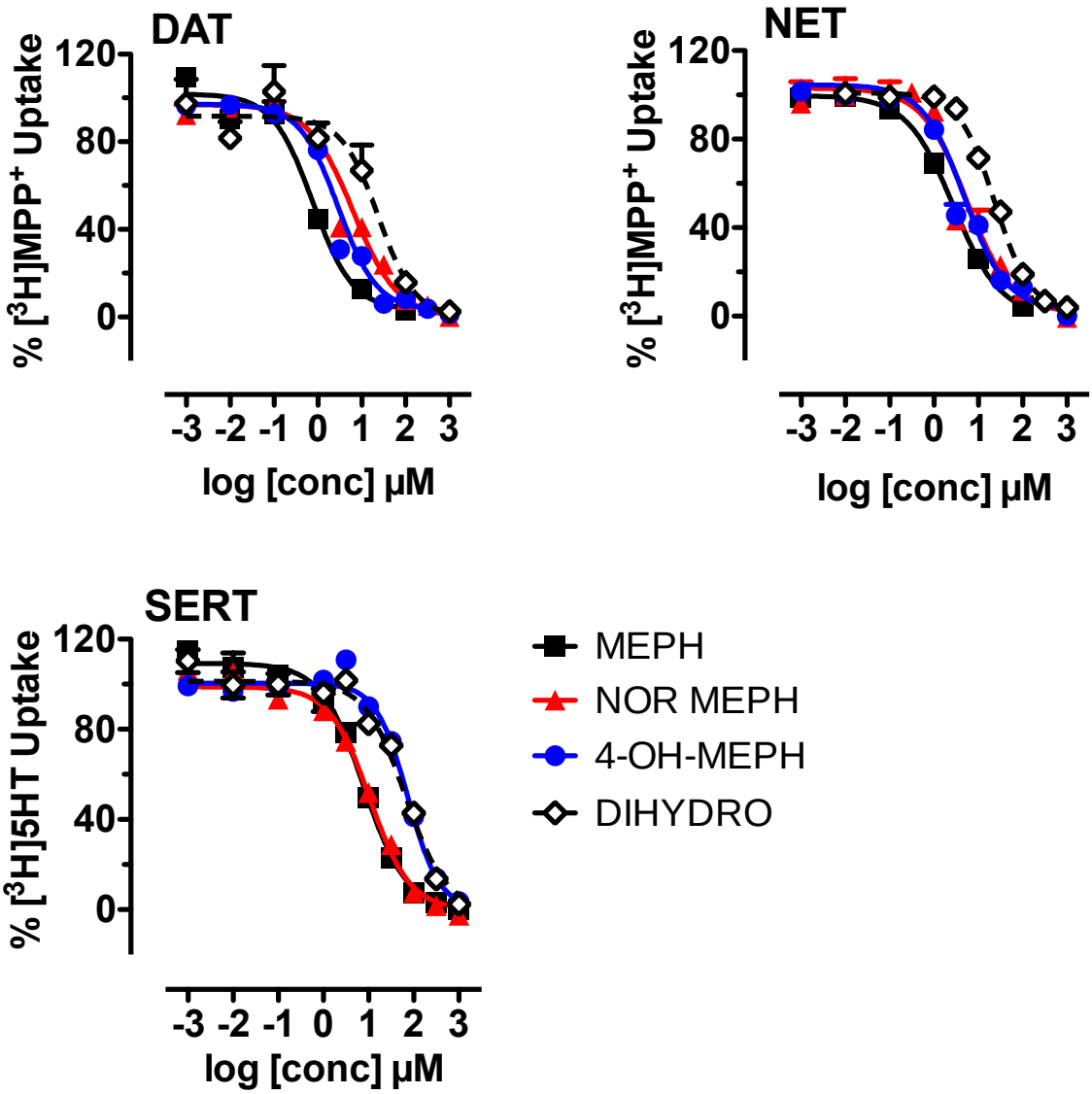

Figure 2. Effects of MEPH, NOR-MEPH, 4-OH-MEPH and DIHYDRO-MEPH on transporter-mediated uptake in HEK293 cells expressing hDAT, hNET and hSERT. Uptake of [^{3}H]-MPP$^+$ via hDAT and hNET, and uptake of [^{3}H]-5HT by hSERT, was performed as described in "Methods"; all symbols represent mean values ± SEM and the numbers in the brackets indicate the number of individual experiments performed in triplicate: hDAT: MEPH (3), NOR-MEPH (4), 4-OH-MEPH (4), DIHYDRO-MEPH (3); hNET: MEPH (4), NOR-MEPH (4), 4-OH-MEPH (3), DIHYDRO-MEPH (4); hSERT: MEPH (3), NOR-MEPH (3), 4-OH-MEPH (3), DIHYDRO-MEPH (3).

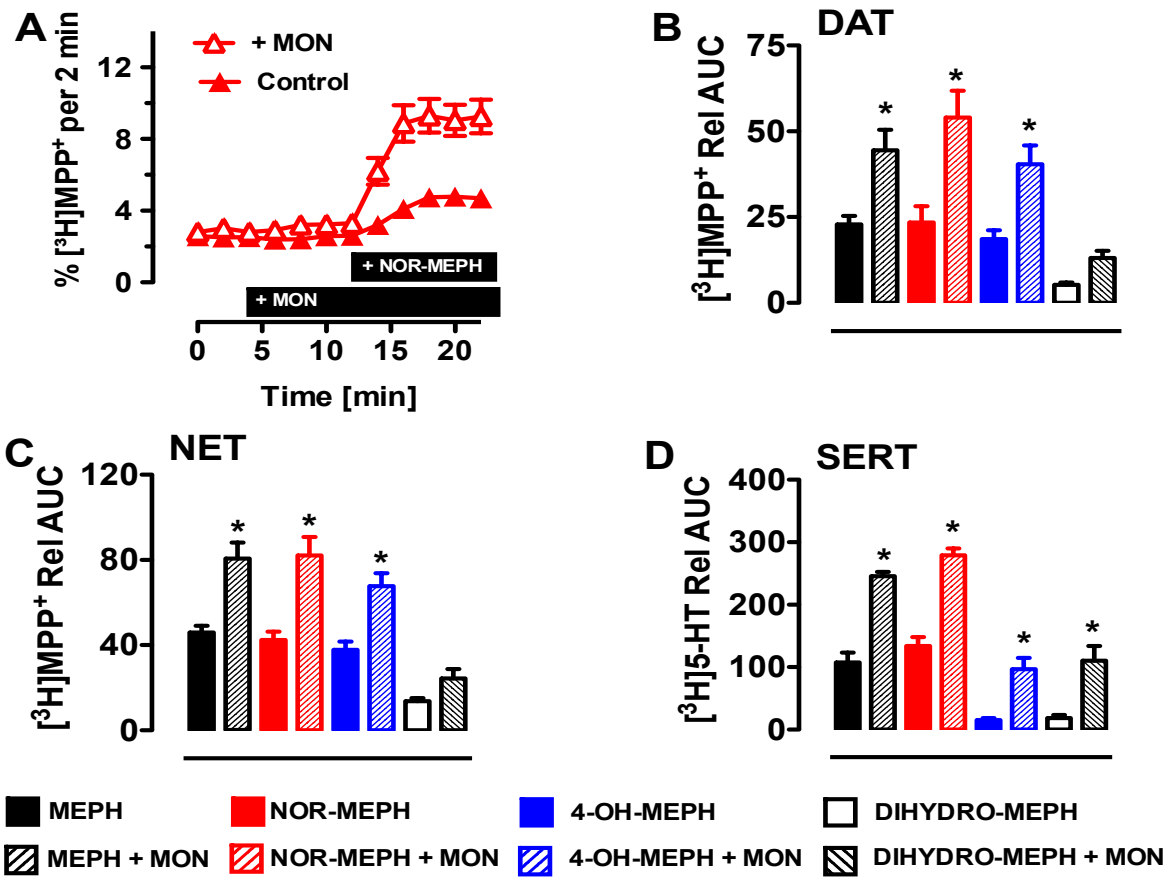

Figure 3. Effects of MEPH, NOR-MEPH, 4-OH-MEPH and DIHYDRO-MEPH on transporter-mediated release of preloaded radiolabeled substrate in cells HEK293 expressing hNET, hDAT and hSERT. [^{3}H]-MPP$^+$ was used as the radiolabeled substrate for hDAT and hNET while release by hSERT-expressing cells was performed using [^{3}H]-5HT as the radiolabeled substrate. [A] Representative experiment showing the effect of NOR-MEPH (10 µM) in the presence or absence of monensin (10 µM) on DAT-mediated efflux of preloaded [^{3}H]-MPP$^+$ (presence of substances indicated by black bar; n=5 independent experiments performed in triplicate). [B to D] For each transporter, area-under-the-curve (AUC) was calculated from 9 fractions collected after drug treatment (10 µM) in the absence or presence of monensin (MON, 10 µM). Solid bars indicate vehicle + drug, whereas hatched bars indicate MON + drug. Bars represent mean values ± SEM and the numbers in the brackets indicate the number of individual experiments performed in triplicate: hDAT: MEPH (6), NOR-MEPH (5), 4-OH-MEPH (5), DIHYDRO-MEPH (5); hNET: MEPH (5), NOR-MEPH (5), 4-OH-MEPH (6), DIHYDRO-MEPH (5); hSERT: MEPH (5), NOR-MEPH (5), 4-OH-MEPH (5), DIHYDRO-MEPH (5). * = $p < 0.05$ (Bonferroni's) compared to corresponding vehicle + drug group.

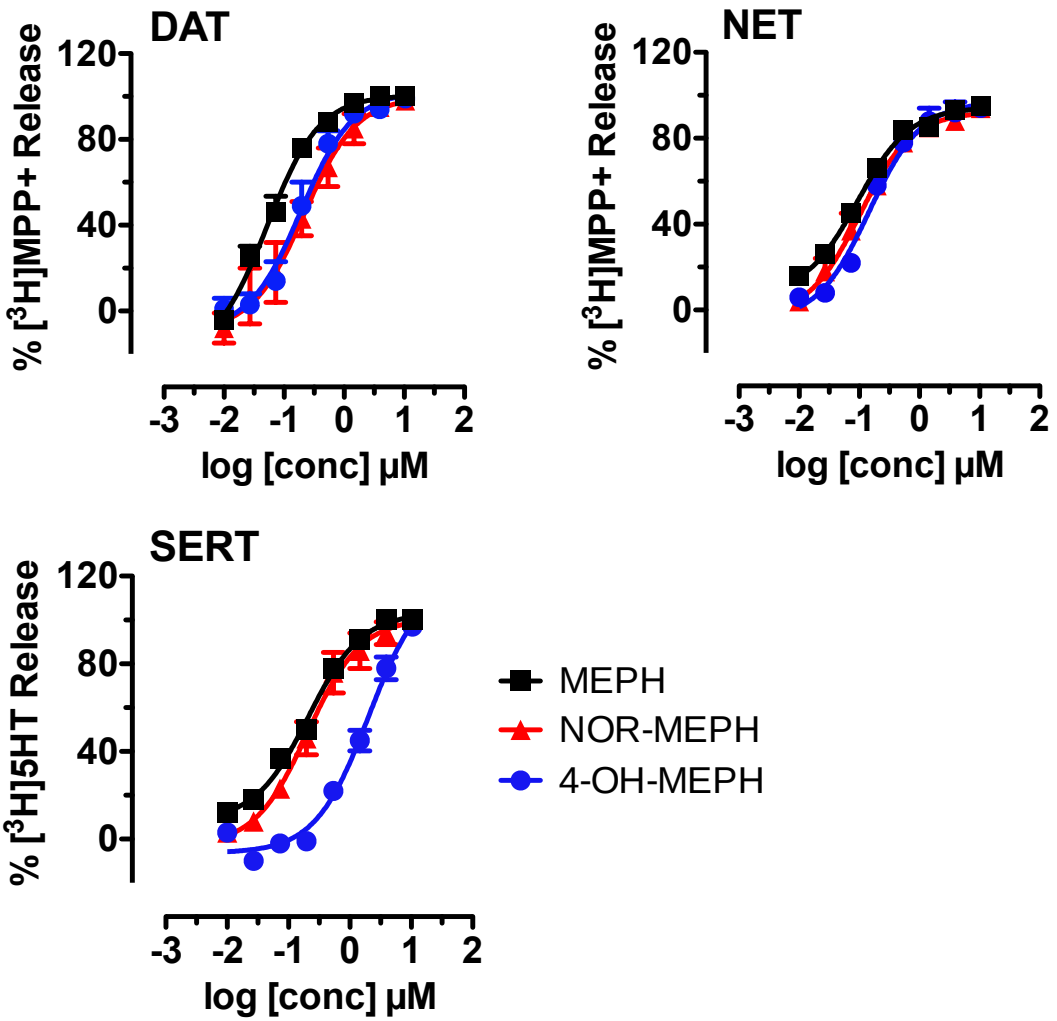

Figure 4. Effects of MEPH, NOR-MEPH and 4-OH-MEPH on transporter-mediated release of preloaded radiolabeled substrate in rat brain synaptosomes. [^{3}H]-MPP$^+$ was the radiolabeled substrate for DAT and NET assays while [^{3}H]-5HT was the radiolabeled substrate for SERT assays. Symbols represent mean values ± SEM obtained from three individual experiments performed in triplicate.

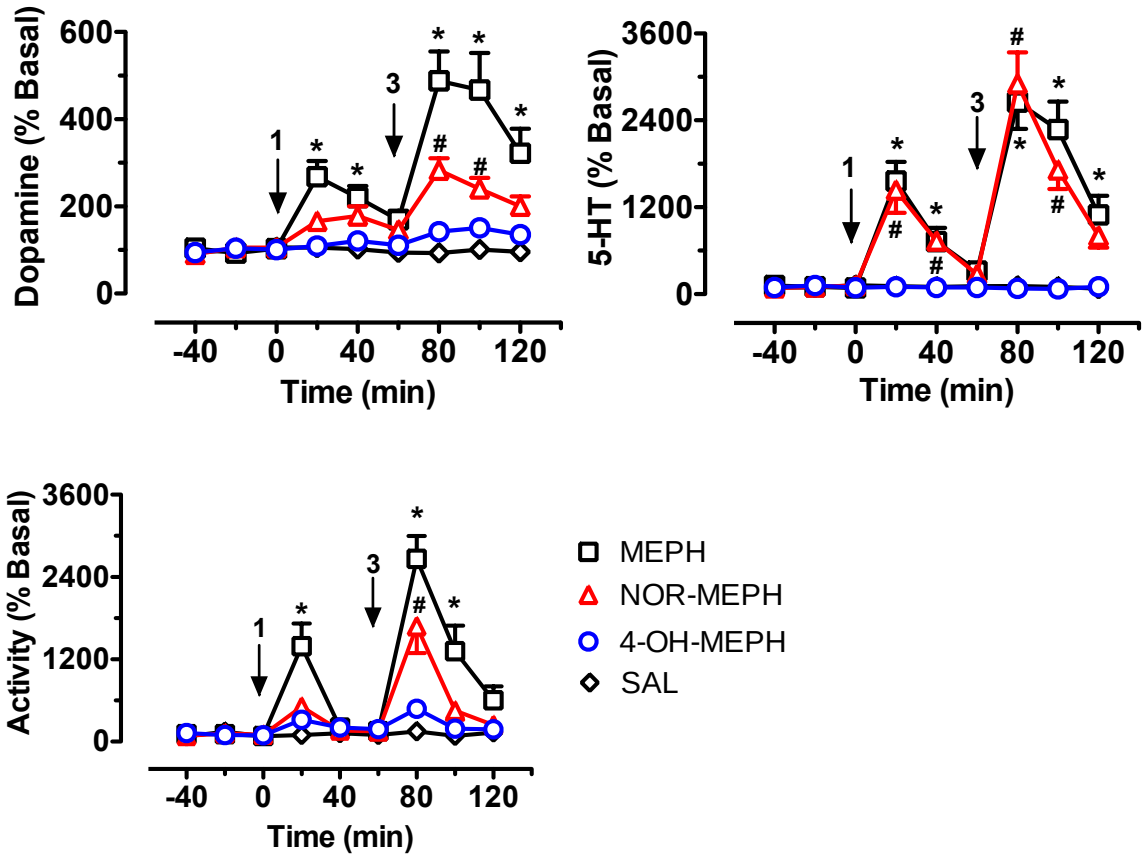

Figure 5. Effects of i.v. administration of MEPH, NOR-MEPH, 4-OH-MEPH or saline (SAL) on neurochemistry and behavior in rats undergoing microdialysis in nucleus accumbens. Drugs were administered intravenously at 1 mg kg^{-1} at time zero, followed by 3 mg kg^{-1} 60 min later. Dopamine and 5-HT were detected by HPLC-EC as described in Methods. Forward locomotion (Activity) was determined by photo-beam breaks. Data are presented as mean ± SEM, *n*=6 rats in the control group (SAL) and *n*=7 rats for all other groups (MEPH, NOR-MEPH and 4-OH-MEPH), arrows indicate time of drug administration. Individual symbols represent significant differences from saline-treated control at corresponding time points ($p<0.05$; Bonferroni's): * denotes significance of MEPH over saline, # denotes significance of NOR-MEPH over saline.

Tables:

Two Tables

	IC_{50} (µM)		
	DAT	**NET**	**SERT**
MEPH	**0.77 (0.53-1.08)**	**2.77 (1.92 -3.97)**	**7.83 (6.32 – 9.75)**
NOR-MEPH	**6.35 (4.66 -8.64)**	**5.46 (3.58 – 8.31)**	**10.61 (9.06 – 12.43)**
4-OH-MEPH	**2.92 (2.35 – 3.6)**	**4.85 (3.28 – 7.17)**	**73.53 (62.5 – 86.51)**
DIHYDRO-MEPH	**23.97 (8.65 – 66.46)**	**23.53 (19.8 – 27.97)**	**64.98 (50.66 – 83.37)**

Table 1: IC_{50} values of test drugs on uptake mediated by hDAT, hNET and hSERT, stably expressed in HEK293 cells. Data are represented as the mean and 95 % confidence intervals in brackets obtained from nonlinear regression fits as shown in Figure 2.

Table 2: EC_{50} values of test drugs on monoamine transporter mediated efflux

	EC_{50} (µM)		
	DAT	**NET**	**SERT**
MEPH	**0.052 (0.036-0.075)**	**0.09 (0.08-0.11)**	**0.21 (0.17 – 0.26)**
NOR-MEPH	**0.22 (0.14 -0.32)**	**0.1 (0.08-0.13)**	**0.21 (0.13 – 0.32)**
4-OH-MEPH	**0.19 (0.13 – 0.267)**	**0.15 (0.11 – 0.19)**	**2.01 (1.390 – 2.91)**

Table 2: EC_{50} values of test drugs on transporter mediated efflux obtained in rat brain synaptosomes. Data are presented as the mean and 95 % confidence intervals in brackets obtained from nonlinear regression fits as shown in Figure 3.