‘Second-Generation’ Mephedrone Analogs, 4-MEC and 4-MePPP, Differentially Affect Monoamine Transporter Function

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INTRODUCTION

In recent years, there has been an alarming increase in the nonmedical use of synthetic psychoactive compounds described as ‘designer drugs’ or ‘legal highs’ (Rosenbaum et al, 2012). These substances are synthesized by rogue chemists who hijack the medical and patent literature to identify structures that target specific neuronal receptors or transporters known to mediate psychoactive effects (Lewin et al, 2014). Synthetic cathinones are designer drugs given innocuous names like ‘bath salts’, ‘plant food’, or ‘research chemicals’ as a ploy to skirt the regulations governing the sale of psychoactive substances (Baumann et al, 2013a; De Felice et al, 2014). 4-Methyl-N-methylcathinone (mephedrone) and 3,4-methylenedioxypyrovalerone (MDPV) are examples of synthetic cathinones that produce stimulant-like subjective effects at low doses, but dangerous side effects after high doses or chronic use (Dargan et al, 2011; Spiller et al, 2011). Adverse consequences include hypertension, tachycardia, anxiety, hallucinations, psychosis, and even death. Because of public health risks, legislative authorities have banned mephedrone, MDPV, and certain other synthetic cathinones in the United States (Drug Enforcement Administration (DEA), Department of Justice, 2013), and similar legislation has been enacted in European countries. In response to legislative bans, enterprising chemists have synthesized a number of ‘second-generation’ replacement analogs as a means to evade regulatory control, and this trend is expected to continue (Leffler et al, 2014; Marinetti and Antonides, 2013).

Like other stimulant drugs, synthetic cathinones exert their pharmacological effects by disrupting the function of solute carrier SLC6 transporter proteins (ie, monoamine transporters) expressed on nerve cells in the brain and periphery (Baumann et al, 2013a; Hadlock et al, 2011; Lopez-Arnau et al, 2012). Monoamine transporters normally mediate the sodium-dependent reuptake of monoamine neurotransmitters, and there are specific transporter proteins for norepinephrine (NET), dopamine (DAT),
MATERIALS AND METHODS

Drugs and Reagents

For uptake and release assays in synaptosomes, [3H]dopamine, [3H]5-HT, and [3H]1-methyl-4-phenylpyridinium ([3H]MPP+) were purchased from DuPont New England Nuclear (Boston, MA). All reagents, buffer salts, and chemicals were obtained from Sigma Chemical (St Louis, MO) unless otherwise noted. Reagents used in the experiments for uptake and efflux assays in cells were purchased and used according to previous work (Hofmaier et al., 2014). Plasmids encoding human SERT were a generous gift of Dr Randy D Blakely.

Animals and Housing

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 250–350 g were housed in standard conditions (lights on 0700–1900 h) with food and water freely available. Rats were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and experiments were performed in accordance with the Institutional Care and Use Committee of the NIDA IRP. Xenopus laevis frogs (Nasco, Fort Atkinson, WI) were kept in aquaria on a strict 12 h light/dark schedule with food available once weekly.

Uptake and Release Assay in Rat Brain Synaptosomes

Uptake and release assays were carried out in rat brain synaptosomes as previously described (Baumann et al., 2013b). Synaptosomes were prepared from rat striatum for DAT assays, whereas synaptosomes were prepared from whole brain minus striatum and cerebellum for SERT assays. For uptake inhibition assays, 5 nM [3H]dopamine and [3H]5-HT were used to assess transport activity at DAT and SERT, respectively. The selectivity of uptake assays was optimized for a single transporter by including unlabeled blockers to prevent uptake of [3H]transmitter by competing transporters. Uptake inhibition assays were initiated by adding...
100 μl of tissue suspension to 900 μl Krebs-phosphate buffer containing test drug and [3H]transmitter. Uptake inhibition assays were terminated by rapid vacuum filtration through Whatman GF/B filters, and retained radioactivity was quantified by liquid scintillation counting. For release assays, 9 nM [3H]MPP + was used as the radiolabeled substrate for DAT, whereas 5 nM [3H]5-HT 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 release assays was optimized for a single transporter by including unlabeled blockers to prevent the uptake of [3H]MPP + or [3H]5-HT by competing transporters. Synaptosomes were preloaded with radiolabeled substrate 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 and retained radioactivity was quantified as described for uptake inhibition.

**In Vivo Microdialysis in Rat Nucleus Accumbens**

Microdialysis procedures were carried out as previously described (Baumann et al., 2012). Male rats were surgically prepared with 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). After a 7–10-day recovery period, catheters were attached to extension tubes and 0.5 × 2 mm microdialysis probes (CMA/12, Harvard Apparatus, Holliston, MA) were inserted into guide cannulae. Ringer’s solution was perfused through the probe at 0.5 μl/min and dialysate samples were collected at 20 min intervals. Drug treatments were given after three stable baseline samples were obtained. Rats received two i.v. drug injections, 1 mg/kg at time 0 followed by 3 mg/kg 60 min later. Control rats received two i.v. saline injections on the same schedule. Concentrations of 5-HT and dopamine were quantified using high-pressure liquid chromatography coupled to electrochemical detection (HPLC-ECD). Dialysate samples were injected onto a microbore HPLC column coupled to an EC detector with a glassy carbon electrode set at +650 mV relative to Ag/AgCl reference. Mobile phase was pumped at 60 μl/min. Chromatographic data were exported to an Empower software system (Waters, Milford, MA) for peak identification, integration, and analysis.

**Uptake and Release Assay in HEK293 Cells**

The uptake and release assays in HEK293 cells were carried out as previously described (Hofmaier et al., 2014). For uptake assays, cells were washed twice with Krebs HEPES buffer. Test drugs were added to cells for 5 min allowing equilibration with transporters. Subsequently, [3H]5-HT and [3H]dopamine were added, and the reaction was stopped after allowing uptake for 1 min. The uptake was terminated by washing with 500 μl of ice-cold Krebs HEPES buffer, cells were lysed with 500 μl of 1% sodium dodecyl sulfate, and tritium was counted on a Packard 2300 TR TriCarb Liquid Scintillation Analyzer. For release studies, HEK293 cells expressing hSERT or hDAT were grown overnight on round glass coverslips (5-mm diameter, 40 000 cells per coverslip) placed in a 96-well plate and preloaded with 0.4 μM [3H]5HT or 0.03 μM [3H]MPP + for 20 min at 37 °C in a final volume of 0.1 ml/well. Coverslips were transferred to small chambers (0.2 ml) and superfused with Krebs HEPES buffer (25 °C, 0.7 ml/min). The 40 min baseline for efflux of radioactivity was followed by addition of test drugs and collection of fractions every 2 min. The experiment was terminated by lysis of the cells with 1% sodium dodecyl sulfate and counted.

**Electrophysiological Recordings in X. laevis Oocytes**

Electrophysiology recordings were performed as recently described (Baumann et al., 2014). Briefly, the plasmid containing hSERT was linearized and in vitro transcription was carried out using a T7 RNA polymerase Kit mMessage mMachne (Ambion, Life Technologies, Grand Island, NY). Stage V–VI oocytes were obtained from *X. laevis* and transferred to calcium-free Ringer’s solution. The oocytes were separated into smaller lobes containing 3 to 5 oocytes and defolliculated by enzymatic digestion with collagenase from *Clostridium histolyticum* (1 mg/ml) for 60 min. Oocytes were selected and transferred to Ringer’s solution. Oocytes were kept at 18 °C in Ringer’s solution containing 2.5 mM sodium pyruvate, 100 μg/ml penicillin, and 100 μg/ml streptomycin. In each oocyte, 10 ng of the prepared hSERT RNA was microinjected. The oocytes were maintained for 7–10 days for functional studies, and solution was changed twice daily. A CA-1B high-performance oocyte clamp was employed for the measurements. The recorded signal was digitized with Digidata 13222A (Axon Instruments, Molecular Devices, Sunnyvale, CA). An Intel PC running pCLAMP 9.2 (Axon Instruments) was used for acquisition. Borosilicate glass capillaries were pulled to a final resistance of 0.4–1.2 MΩ and filled with 3 M KCl. Oocytes were impaled and the membrane potential was clamped to a holding potential of −60 mV. For continuous superfusion with ND100 solution (100 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, pH adjusted to 7.4 with NaOH), a gravity-driven superfusion system was used. Recordings were started after a stable current baseline was established. The current was sampled with 100 Hz and low pass filtered with 20 Hz.

**Ligand and Protein Model Preparation**

The ligand structures were built as (S)-enantiomers in protonated form using the software MOE (Molecular Operating Environment (MOE), Montreal, QC, Canada). The recently crystallized *Drosophila* DAT, in the outward facing conformation in complex with nortriptyline (dDAT<sub>cryst</sub>), was used as a template for transporter modeling (Pennatsa et al., 2013). dDAT displays close to 70% sequence homology with hSERT, hDAT, and hNET in the substrate-binding pocket. Sequence alignment was performed using ClustaX (Thompson et al., 2002). Nonstructural waters were removed from the dDAT<sub>cryst</sub> structure and 250 homology models of each of the human transporters in complex with nortriptyline were created using Modeller 9.11 (Sali et al., 1995). Nortriptyline has dissociation constants (K<sub>D</sub>) of 18 nM at hSERT, 1140 nM at hDAT, and 4.4 nM at hNET (Tatsumi et al., 1997). The models with the highest ‘Discrete Optimization of Protein Energy’ (DOPE) score showed no...
disallowed dihedrals near the central binding site and were protonated at pH 7 using the Protonate3D tool in MOE. Nortriptyline and residues within a radius of 5 Å were energy minimized using a distance-dependent dielectric constant of 2 (Hou et al, 2011) to 80 in the OPLS-AA force field (Jorgensen et al, 1996).

Docking of 4-MEC and 4-MePPP

In order to determine the influence of the SERT Thr439 conformation on ligand placement, the side chain was rotated by 180° along its Cα-Cβ bond. The binding site topology was optimized by energy minimization of nortriptyline (or Thr439 in SERT) as noted above for dDAT (Hou et al, 2011; Jorgensen et al, 1996). The transporter–ligand complexes were loaded into the docking software GOLD 5.2 (Jones et al, 1997) that uses a genetic algorithm to obtain poses nondeterministically. Waters were removed and the binding site was defined as the center of mass of the inhibitor. The cathinone substructure was used for setting restraints, whereby the cationic nitrogen was forced to be within 2–4 Å to the Tyr95/Phe76 (SERT/DAT) backbone carbonyl oxygen. This is in analogy to the positive partial charge density of antidepressant ligands in dDATcryst and in the humanized leucine transporter structures (Wang et al, 2013). Binding modes were generated 50 times per ligand using GoldScore with maximum search efficiency, and the poses retrieved were clustered based on their placement into specific subpockets.

Data Analysis and Statistics

For uptake and release assays, the data from three experiments were fit to a dose–response curve equation, and IC50 or EC50 values were calculated using GraphPad Prism. For microdialysis experiments, the first three samples collected were considered baseline samples and all subsequent monoamine measures were expressed as a percentage of the mean of this baseline. Effects of drugs on dialysate 5-HT and dopamine were evaluated using two-way ANOVA (treatment × time) followed by Bonferroni post hoc tests at specific time points after drug injection. For transporter-mediated currents, the comparison of the maximum currents across drugs was analyzed by one-way ANOVA with Tukey’s post hoc test. P < 0.05 was chosen as the minimum criterion for statistical significance.

RESULTS

Effects of 4-MEC and 4-MePPP on DAT and SERT in Synaptosomes

Figure 2 depicts the effects of mephedrone, 4-MEC, and 4-MePPP in transporter assays carried out in rat brain synaptosomes. The ability of test drugs to inhibit uptake of [3H]5-HT and [3H]dopamine is shown in Figure 2a and b, respectively. Mephedrone and 4-MEC displayed nearly equal potency at inhibiting uptake at SERT (IC50 = ~500 nM) and at DAT (IC50 = ~800 nM). In contrast, 4-MePPP was much more potent as an inhibitor at DAT when compared with SERT, with an IC50 = 215 ± 13 nM at DAT vs IC50 = > 10 000 nM at SERT. Thus, mephedrone and 4-MEC are nonselective uptake blockers, whereas 4-MePPP is 40-fold selective for DAT over SERT. As discussed in previous publications (Baumann et al, 2012, 2013a), the effects of drugs in uptake inhibition assays cannot reveal whether drugs are acting as transporter blockers or substrates, and hence we next tested the effects of drugs in the synapsosome release assay. The ability of test drugs to evoke release of preloaded [3H]5-HT from SERT (c) and [3H]MPP+ from DAT (d) evoked by 4-MEC, 4-MePPP, and mephedrone. Data are mean ± SD for N = 3 separate experiments performed in triplicate.

Figure 3a demonstrates a main effect of drug treatment on extracellular 5-HT at either dose tested. Figure 3b shows a main effect of drug treatment on extracellular 5-HT at either dose tested. Figure 3a shows a main effect of drug treatment on extracellular dopamine (F2,16 = 27.26, p < 0.0001), the effects of 4-MEC and 4-MePPP on dopamine were opposite to the changes in 5-HT. More specifically, 4-MePPP produced dose-related
increases in extracellular dopamine, with 1 mg/kg producing a 1.8-fold elevation above baseline and 3 mg/kg producing a 3.3-fold elevation. 4-MEC, on the other hand, produced no effect on dopamine at 1 mg/kg, but a 1.9-fold increase after the 3 mg/kg dose. Figure 3c and d demonstrate that drug treatment had main effects on forward locomotion (Motor) (c) and stereotypic movements (Stereo) (d). Data are mean ± SEM expressed as % baseline for N=6-7 rats/group. Arrows indicate time of injections and numbers indicate i.v. mg/kg doses. *P<0.05 compared with saline-injected control at specific time points.

**Effects of 4-MEC and 4-MePPP on Human Transporters Expressed in Cells**

Based on the results from rat experiments, we wished to explore the molecular mechanism of action for 4-MEC and 4-MePPP in greater detail, and hence the effects of these drugs were examined in HEK293 cells stably expressing human transporters. Figure 4a and b show the effects of test drugs on uptake inhibition in cells expressing human SERT (hSERT) or human DAT (hDAT), respectively. In agreement with findings from synaptosomes, 4-MEC showed nonsel ective inhibition of uptake, with IC$_{50}$ values of $10.9 \pm 2.2 \mu$M at hSERT and $3.9 \pm 0.4 \mu$M at hDAT. In contrast, 4-MePPP displayed much higher potency at inhibiting uptake at hDAT (IC$_{50} = 1.08 \pm 0.1 \mu$M) when compared with hSERT (IC$_{50} = 126 \pm 36 \mu$M). Next, we compared the effects of 4-MEC and 4-MePPP on transporter-mediated efflux using superfusion methods. In these experiments, the time-dependent efflux of [³H]5-HT through hSERT and [³H]MPP+ through hDAT was assessed in the presence or absence of monensin (10 $\mu$M), an ionophore that dissipates the normal Na+ gradient across cell membranes and selectively enhances the efflux caused by transporter substrates (Baumann et al, 2013b; Scholze et al, 2000). Thus, monensin can be used to discriminate the effects of transporter substrates vs blockers. Figure 4c shows that 4-MEC (10 $\mu$M) induced efflux of [³H]5-HT but 4-MePPP did not. Importantly, the efflux of [³H]5-HT produced by 4-MEC was dramatically enhanced in the presence of monensin, confirming that 4-MEC is a substrate at hSERT. Figure 4d demonstrates that 4-MEC and 4-MePPP both induced modest efflux of [³H]MPP+ at hDAT, but in neither case was this response altered by monensin.

**Effects of 4-MEC and 4-MePPP on SERT-Mediated Currents**

As a final test to confirm that 4-MEC and 4-MePPP display differential effects at SERT, we examined transporter-mediated currents in oocytes expressing hSERT (Baumann et al, 2014). For these experiments, the effects of drugs were only evaluated in SERT-expressing cells as neither drug elicited substrate activity at DAT. Figure 5a and b depict the effects of 4-MEC and 4-MePPP on SERT-mediated currents, respectively. 4-MEC evoked robust dose-related inward currents that followed a bell-shaped dose response. The greatest magnitude of current produced by 4-MEC (30 $\mu$M) was nearly equivalent to that produced by 10 $\mu$M 5-HT. Consistent with the profile of a transporter blocker, 4-MePPP did not elicit any transporter mediated-current at doses up to 100 $\mu$M. Figure 5c shows the current-inducing effects of 4-MEC and 4-MePPP when normalized to the effects of 10 $\mu$M 5-HT. This figure highlights the bell-shaped dose response for 4-MEC and the lack of effect for 4-MePPP.

**Computational Docking with 4-MEC and 4-MePPP**

The differential effects of 4-MEC and 4-MePPP at SERT prompted us to employ a molecular docking approach to explore the structural peculiarities between SERT and DAT, and possible differences in the binding modes of the two compounds. The binding modes of the two cathinones in the hSERT and hDAT homology models were analogous to the LeuBAT inhibitor binding mode (Wang et al, 2013), whereby the aromatic rings are placed in the previously reported subpocket B (Andersen et al, 2010; Seddik et al, 2013). 4-MEC and 4-MePPP only differ in their N-substitution, with 4-MePPP having a sterically more demanding substituent (pyrrolidine ring vs ethyl chain). Remarkably, this difference causes a >100-fold decrease in the affinity of 4-MePPP for SERT vs DAT. It seems unlikely that this is solely due to differences in the interaction of the cathinone nitrogen with distinct amino acids, as the only difference in the binding site is F76/Y95 (Table 1). Figure 6 depicts docking poses of 4-MEC and 4-MePPP in hDAT (Figure 6a) and hSERT (Figure 6b and c). Although both compounds show analogous poses in hDAT, which is
supported by their similar effects at inhibiting [3H]dopa-
mine uptake, the placement in hSERT is less consistent.
Importantly, this inconsistency only affects 4-MePPP, which
shows two clusters, with the aromatic moiety being placed
either deeper in subpocket B or in subpocket C. The latter
configuration seems unlikely, as no transporter inhibitor
was found to occupy this site in LeuBATs. Thus, the
ambiguous positioning of 4-MePPP in hSERT might be
because of its larger N-pyrrolidino substituent that forces a
slight shift deeper into subpocket B when compared with
the case for 4-MEC. In the case of subpocket B of hDAT,
there is sufficient space to accommodate 4-MePPP because
of the smaller side chains present (ie, Ala423, Val152, and
Gly153); subpocket B in hSERT is formed by bulkier amino-
acid side chains (Thr439, Ile172, and Ala173). In particular,
Thr439 might be responsible for steric repulsion of 4-
MePPP in hSERT, rendering the complex less stable. Thus,
two different side chain conformations of Thr439 were
probed and similar docking patterns were found (Figure 6b
and c), whereby either the polar hydroxyl group or a
proximal methyl group repels the positioning of 4-MePPP.
In addition, reducing the flexibility of Thr439 upon binding
of 4-MePPP also leads to an entropically unfavorable
contribution to the binding free energy.

DISCUSSION
A major goal of our study was to determine the mechanism
of action and pharmacological effects of the mephedrone
analogs, 4-MEC and 4-MePPP. In previous publications, we
and others have shown that mephedrone is a nonselective
substrate for monoamine transporters, thereby causing the
release of 5-HT, dopamine, and norepinephrine (Baumann
et al, 2012; Eshleman et al, 2013; Simmler et al, 2013). Once
legislation was enacted to render mephedrone illegal, 4-
MEC and 4-MePPP began appearing in the recreational
drug marketplace (Ayres and Bond, 2012; Brandt et al, 2011;
Leffler et al, 2014), and 4-MEC has been associated with
adverse medical consequences leading to death (Gil et al,
2013; Rojek et al, 2014). The present in vitro findings from
rat brain synaptosomes show that 4-MEC displays unique
activity as a SERT substrate/DAT blocker, whereas 4-MePPP
is a DAT blocker with little activity at SERT. Consistent
with synaptosome data, i.e. administration of 4-MEC/4-MePPP.
Data are mean ± SD for n = 3 separate experiments.

Figure 4. Inhibition of uptake and stimulation of efflux produced by 4-MEC and 4-MePPP in HEK293 cells stably expressing hSERT and hDAT. Inhibition of
[3H]-5-HT uptake by hSERT (a) and [3H]-DA uptake by hDAT (b) in cells. Efflux of [3H]-5-HT (c) and [3H]-MPP+ (d) via hSERT and hDAT, respectively.
Effects of 10 μM 4-MEC and 10 μM 4-MePPP on efflux were carried out in the presence of Krebs HEPES buffer (KHP) or 10 μM monensin. Arrows show the
administration of 4-MEC/4-MePPP. Data are mean ± SD for n = 3 separate experiments.
activity at SERT and blocker activity at DAT, a profile that we call 'hybrid' transporter activity. Simmler et al. (2014) reported data consistent with our findings, showing 4-MEC is a 5-HT releaser but a blocker at hDAT and hNET. Although this profile is unusual, it has been reported previously (Blough et al., 2014; Simmler et al., 2014; Yu et al., 2000). Yu et al. (2000) described SERT substrate/DAT blocker activity for N-ethylaminopropiophenone (ie, N-ethylcathinone), a bioactive metabolite of the clinically available appetite suppressant diethylpropion. 4-MEC and N-ethylcathinone display similar chemical structures, and hence it appears that extension of the N-alkyl chain of cathinone compounds, from N-methyl to N-ethyl, is sufficient to convert activity at DAT from a substrate (eg, mephedrone) to a blocker (eg, 4-MEC). In contrast, mephedrone and 4-MEC display nearly equivalent effects.

Table 1 Residues Surrounding the Cationic Nitrogen of Cathinone-Type Ligands in Human SERT and DAT

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Abbreviations: A, alanine; D, aspartate; F, phenylalanine; G, glycine; S, serine; Y, tyrosine.

Figure 5 Dose–response effects of 4-MEC and 4-MePPP on SERT-generated currents in Xenopus oocytes. Representative electrophysiological traces for 4-MEC (a) and 4-MePPP (b). Concentration–response curves, pooled from different oocytes for 4-MEC and 4-MePPP (c). Data in (c) are mean ± SEM for n = 8 oocytes from two independent preparations. Maximal current for 4-MEC was measured at 30 μM and no current was observed for 4-MePPP.

Figure 6 Central binding site of (a) DAT with docking poses of 4-MEC (cyan) and 4-MePPP (orange), (b) SERT with the Thr439 hydroxyl pointing toward the S1 site, and (c) SERT with the Thr439 methyl pointing toward the S1 site. The poses of both ligands in DAT are very consistent whereby the 4-methyl group points into subsite B. The same consistency is found in SERT for 4-MEC, but not for 4-MePPP, presumably because of an unfavorable fit into this subpocket forced by the larger substituent on the nitrogen atom.
as 5-HT releasers, suggesting that minor changes in N-alkyl chain length do not alter substrate activity at SERT. The data with 4-MEC and N-ethylcathinone illustrate the close structural resemblance among synthetic cathinones in the ‘street drug’ marketplace and those being prescribed clinically, such as diethylpropion and bupropion (Carroll et al., 2014; Cercato et al., 2009). Indeed, Blough et al. (2014) have proposed that hybrid transporter compounds based on the N-cyclopropylcathinone structure could have value in the treatment of substance use disorders. Future studies should be carried out to examine the possible therapeutic potential of hybrid transporter ligands.

Extending the N-alkyl chain of 4-MEC to form the pyrrolidine ring structure of 4-MePPP has marked effects on pharmacology, converting the compound to a DAT-selective transporter blocker. In fact, the in vitro pharmacology of 4-MePPP resembles that of pyrrolidinophenone compounds like pyrovalerone and MDPV rather than mephedrone (Baumann et al., 2013b; Cameron et al., 2013; Kolanos et al., 2013; Marusich et al., 2014). Meltzer et al. (2006) reported that pyrovalerone analogs are potent blockers at DAT and NET with little influence on SERT. More recently, we demonstrated that MDPV, α-PVP, and related pyrrolidinophenones are potent and selective blockers at DAT and NET in rat brain synaptosomes (Baumann et al., 2013b; Marusich et al., 2014). Kolanos et al. (2013) examined the effects of MDPV and α-PVP in Xenopus oocytes expressing hDAT and found that these compounds do not induce inward DAT-mediated currents, consistent with the present results showing that 4-MePPP does not display DAT substrate activity. Collectively, the in vitro data indicate that cathinone analogs that possess a pyrrolidine ring structure, or perhaps other bulky N-alkyl substituents, will function as selective uptake blockers at DAT and NET.

The present microdialysis results show that transporter activity of 4-MEC and 4-MePPP strongly influences the in vivo neurochemical and behavioral effects of the drugs. Administration of 4-MEC to rats produces predominant increases in extracellular 5-HT with small increases in extracellular dopamine, consistent with its hybrid transporter actions. The in vivo neurochemical profile of 4-MEC mimics the effects of mephedrone and methylene (Baumann et al., 2012; Kehr et al., 2011; Wright et al., 2012), but 4-MEC is less potent and has weaker effects on dopamine when compared with other ring-substituted cathinones. Previous studies have shown that 5-HT-releasing actions of amphetamines and cathinones can dampen dopamine-mediated locomotor and reinforcing effects in rats (Baumann et al., 2011; Bonano et al., 2014), and hence it might be predicted that 4-MEC has weak stimulant properties. In contrast, 4-MePPP produces selective increases in extracellular dopamine and robust locomotor activation. The in vivo neurochemical profile of 4-MePPP mimics the effects of MDPV (Baumann et al., 2013b), but 4-MePPP is 10-fold less potent and its effects are short-lived. It is well established that elevations in extracellular dopamine in the nucleus accumbens are correlated with the magnitude of locomotor activation produced by stimulant drugs (Baumann et al., 2011; Zolkowska et al., 2009), and the hyperactivity produced by 4-MePPP agrees with the reported locomotor effects of MDPV and related pyrrolidinophenones (Aarde et al., 2013b; Fantegrossi et al., 2013; Gatch et al., 2013; Marusich et al., 2014; Marusich et al., 2012). The selective increase in extracellular dopamine produced by 4-MePPP suggests that this drug will be readily self-administered (Aarde et al., 2013b; Watterson et al., 2014).

Our experiments in synaptosomes provide the advantage of rapid drug screening in native tissue, whereas experiments in cells allow a more detailed assessment of drug–transporter interactions. Here we examined the effects of 4-MEC and 4-MePPP in cells expressing hDAT and hSERT. In uptake inhibition assays, 4-MEC acts as a nonselective transporter blocker whereas 4-MePPP is selective for hDAT, in agreement with the findings in synaptosomes. It is noteworthy that IC50 values for 4-MEC and 4-MePPP in transporter-expressing cells are somewhat higher (ie, apparent lower potency) than those determined in synaptosomes. Differences in assay procedures used for synaptosomes vs cells could explain these differences. In addition, the complement of accessory membrane proteins present in synaptosomes may not be present in nonneuronal cell systems (Wilhelm et al., 2014), and this could influence absolute potency values. In the superfusion assays, 4-MEC evokes 5-HT efflux whereas 4-MePPP does not. The 5-HT efflux produced by 4-MEC is markedly potentiated by monensin, an ionophore that dissipates Na+ gradients across cell membranes enhancing intracellular Na+ (Hofmaier et al., 2014). We have shown previously that monensin augments transporter-mediated efflux caused by substrates but not blockers (Baumann et al., 2013b; Hofmaier et al., 2014; Scholze et al., 2000), and hence the findings with monensin reported here provide decisive evidence that 4-MEC is a SERT substrate. 4-MEC and 4-MePPP also evoke modest efflux from hDAT, but these effects are not altered by monensin, confirming that neither drug is a DAT substrate. Our data from cells expressing human transporters reinforce the findings from rat brain tissue, and serve to validate the translational value of studying cathinone-type drugs in rodent models.

Perhaps the most sophisticated method for examining the interactions of drugs with monoamine transporters involves the measurement of transporter-mediated ionic currents (Sitte et al., 1998; Sonders et al., 1997). Because the SLC6 transporters co-transport Na+ ions along with substrate, inward depolarizing current is generated during translocation of substrate from the outside of the cell to the inside (Kristensen et al., 2011). Thus, measuring the electrophysiological signature of transporter ligands can give direct information about the molecular mechanism of action for these substances. We found that 4-MEC, but not 4-MePPP, induces inward current analogous to prototypical SERT substrates like fenfluramine and p-chloroamphetamine (Baumann et al., 2014; Gobbi et al., 2008). Moreover, the current dose–response relationship with 4-MEC appears bell shaped, similar to the effects of other transporter substrates. The decrease in current measured at the highest concentration of 4-MEC (ie, 100 μM) is likely because of the intracellular accumulation of substrate that tends to inhibit SERT-mediated current (Adams and DeFelice, 2003). We have previously shown that the magnitude of inward current produced by SERT substrates may be involved in the persistent 5-HT depletions caused by these drugs in rats.
(Baumann et al, 2014; Gobbi et al, 2008). Our electrophysiological findings with 4-MEC suggest that future studies should examine the potential for this drug to produce long-term serotonergic deficits in rodent models.

Based on the present structure–activity data, a crucial question arises: how does extending the N-alkyl chain of mephedrone produce major changes in pharmacology? We carried out molecular modeling studies to address this question, with specific reference to the structural differences between 4-MEC (N-ethyl) and 4-MePPP (N-containing pyrrolidine ring) that lead to marked loss of activity at SERT for 4-MePPP. Using a computational docking approach based on dDAT crys (Pennmatsa et al, 2013), we show that the binding subpocket B of SERT is smaller because of bulkier amino-acid side chains when compared with the binding subpocket of DAT. Therefore, it is tempting to speculate that SERT is less able to accommodate the bulky pyrrolidine ring structure of 4-MePPP when compared with the smaller N-ethyl chain of 4-MEC. The present modeling approach may have key predictive value when attempting to understand transporter selectivity of new cathinone analogs as they appear in the recreational drug marketplace.

To summarize, the structure–activity data reported here demonstrate that changing the N-alkyl substituent of cathinone drugs can profoundly influence their pharmacology. 4-MEC is a SERT substrate/DAT blocker with predominant 5-HT-releasing effects in vivo. The serotonergic actions of 4-MEC may reduce its stimulant properties but enhance its propensity for producing long-term 5-HT deficits. 4-MePPP is a selective DAT blocker with robust locomotor stimulant effects in vivo. Although our study focused on the effects of drugs on DAT and SERT, it seems likely that 4-MEC and 4-MePPP interact with NET as well (eg, see Simmler et al, 2014). Future studies should examine the neurotoxic potential, abuse liability, and noradrenergic actions of 4-MEC, 4-MePPP, and other newly emerging cathinone derivatives.

FUNDING AND DISCLOSURE

HHS has received honoraria for lectures and consulting from Lundbeck, Ratiopharm, Roche, Sanofi-Aventis, and Serumwerk Bernburg. The other authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by the Austrian Research Fund/FWF grants F3506 and W1252 to HHS and the Intramural Research Program of the NIDA, NIH, grant DA000523-07 to MHB.

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