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The synthetic cathinones, butylone and pentylone, are stimulants that act as dopamine transporter blockers but 5-HT transporter substrates

Kusumika Saha^{1,2}, Yang Li¹, Marion Holy¹, Kurt R. Lehner^{3,4}, Mohammad O. Bukhari^{3,5}, John S. Partilla³, Walter Sandtner¹, Harald H. Sitte^{1,6}, and Michael H. Baumann³

¹Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna, Waehringerstrasse 13A, A-1090 Vienna, Austria

²Institut Cochin, INSERM U1016, CNRS UMR8104, Université Paris Descartes, Sorbonne Paris Cité, 27 rue du Faubourg St-Jacques, 75014, Paris, France

³Designer Drug Research Unit (DDRU), Intramural Research Program (IRP), NIDA, NIH, 333 Cassell Drive, Baltimore, MD 21224, USA

⁴Department of Neurosurgery, Johns Hopkins Hospital, Baltimore, MD 21287, USA

⁵University of Pittsburgh Medical Center, Altoona, PA 16601, USA

⁶Center for Addiction Research and Science, Medical University Vienna, Waehringerstrasse 13A, A-1090 Vienna, Austria

Abstract

Rationale—Synthetic cathinones continue to emerge in recreational drug markets worldwide. 1-(1,3-Benzodioxol-5-yl)-2-(methylamino)butan-1-one (butylone) and 1-(1,3-Benzodioxol-5-yl)-2-(methylamino)pentan-1-one (pentylone) are derivatives of the cathinone compound, 1-(1,3-benzodioxol-5-yl)-2-(methylamino)propan-1-one (methyline), that are being detected in drug products and human casework.

Objectives—The purpose of the present study was to examine the neuropharmacology of butylone and pentylone using *in vitro* and *in vivo* methods.

Methods—*In vitro* uptake and release assays were carried out in rat brain synaptosomes and in cells expressing human dopamine transporters (DAT) and 5-HT transporters (SERT). *In vivo*

Corresponding Author: Michael H. Baumann, Ph.D., Chief, Designer Drug Research Unit, IRP, NIDA, NIH, DHHS, 333 Cassell Drive, Suite 4400, Baltimore, MD 21224, USA, Tel: +1 443 740-2660, mbaumann@mail.nih.gov.

AUTHORSHIP CONTRIBUTIONS

MHB designed and oversaw *in vitro* and *in vivo* experiments in animals. JSP carried out uptake inhibition and release assays in synaptosomes, whereas KRL and MOB carried out microdialysis experiments in rats. HHS designed and oversaw experiments in HEK-293 cells transfected with human DAT and SERT. KS, YL, MH and WS carried out experiments in transfected cells. MHB and HHS analyzed data. KS and MHB wrote the first draft of the paper, and all other authors contributed significantly to the writing of the final manuscript.

CONFLICT OF INTEREST

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microdialysis was performed in the nucleus accumbens of conscious rats to assess drug-induced changes in neurochemistry.

Results—Butylone and pentylone were efficacious uptake blockers at DAT and SERT, though pentylone was more DAT-selective. Both drugs acted as transporter substrates that evoked release of [³H]5-HT at SERT, while neither evoked release at DAT. Consistent with the release data, butylone and pentylone induced substrate-associated inward currents at SERT but not DAT. Administration of butylone or pentylone to rats (1 and 3 mg/kg, i.v.) increased extracellular monoamines and motor activity, but pentylone had weaker effects on 5-HT and stronger effects on motor stimulation.

Conclusions—Our data demonstrate that increasing the α -carbon chain length of methylone creates “hybrid” transporter compounds which act as DAT blockers but SERT substrates. Nevertheless, butylone and pentylone elevate extracellular dopamine and stimulate motor activity, suggesting both drugs possess significant risk for abuse.

Keywords

5-HT; cathinone; dopamine; microdialysis; neurochemistry; transporter

Introduction

The abuse of new psychoactive substances (NPS) is a persistent public health concern (Baumann and Volkow 2016; Logan et al. 2017). NPS can be defined as drugs that are not scheduled under the Single Convention on Narcotic Drugs (1961) or the Convention on Psychotropic Substances (1971) but may pose health risks (Madras 2017). Synthetic cathinones are a major class of NPS that are often found in products labeled as “bath salts” or “research chemicals” (Baumann 2014). The misuse of cathinone derivatives such as 1-(1,3-benzodioxol-5-yl)-2-(methylamino)propan-1-one (methylone) has caused serious adverse effects including psychosis, agitation, violent behaviors, tachycardia, hyperthermia and even death (Spiller et al. 2011; Pearson et al. 2012; Carbone et al. 2013). Preclinical studies in rats show that methylone is a psychomotor stimulant drug (Baumann et al. 2012; Lopez-Arnau et al. 2012; Gatch et al. 2013) that supports self-administration behavior (Watterson et al. 2012; Creehan et al. 2015; Schindler et al. 2016). Methylone exerts its behavioral effects by interacting with plasma membrane transporters for dopamine (DAT), norepinephrine (NET) and 5-HT (SERT) to enhance monoamine transmission in the brain (Baumann et al. 2012; Eshleman et al. 2013; Simmler et al. 2013). More specifically, methylone acts similarly to the club drug 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) as a non-selective transporter substrate, which triggers transporter-mediated release of dopamine, norepinephrine and 5-HT from neurons.

Due to public health concerns, methylone was rendered illegal by temporary legislation in 2011 (Drug Enforcement Administration 2011), and permanently placed into Schedule I control in 2013 (Drug Enforcement Administration 2013). Nevertheless, chemical analogs of methylone, including 1-(1,3-benzodioxol-5-yl)-2-(methylamino)butan-1-one (butylone) and 1-(1,3-benzodioxol-5-yl)-2-(methylamino)pentan-1-one (pentylone), have appeared in recreational drug markets as clandestine chemists try to stay one step ahead of drug control

laws. From a chemical structure perspective, butylone is the α -ethyl analog of methylone whereas pentylone is the α -propyl analog (see Figure 1 for chemical structures). Forensic studies have identified butylone and pentylone in drug products purchased from street vendors (Zuba and Byrska 2013; Leffler et al. 2014) and in human casework (Marinetti and Antonides 2013; Elliott and Evans 2014). Given the structural similarities between methylone, butylone and pentylone, it is tempting to speculate that clinical effects of these drugs might be similar (Prosser and Nelson 2012), but there is limited information in this regard. In one case report, ingestion of butylone in combination with methylone led to severe hyperthermia and 5-HT syndrome resulting in death (Warrick et al. 2012). Due to public health concerns, butylone and pentylone were placed into permanent Schedule I control in 2017 (Drug Enforcement Administration 2017).

Preclinical investigations in rats show that butylone and pentylone are psychomotor stimulants (Lopez-Arnau et al. 2012; Gatch et al. 2015) that support drug self-administration behavior (Dolan et al. 2018; Javadi-Paydar et al. 2018). Not surprisingly, these drugs interact with monoamine transporters in rat brain tissue and in cells expressing human DAT, NET or SERT. Lopez-Arnau and coworkers (2012) first reported that butylone inhibits uptake of [3 H]dopamine and [3 H]5-HT in rat brain synaptosomes, but substrate activity was not tested. Studies carried out in cells expressing human transporters agree that butylone inhibits uptake at DAT, NET and SERT, with potency and selectivity similar to methylone (Eshleman et al. 2013, Simmler et al. 2013). Eshleman et al. (2013) demonstrated that butylone acts as a substrate-type releaser at human SERT, but not at human DAT or NET, suggesting the compound displays “hybrid” transporter activity characterized by a substrate effect at SERT but blocker effects at DAT and NET (see also Blough et al. 2014; Saha et al. 2015). Pentylone appears to display a mechanism of action analogous to butylone in transfected cells (Simmler et al. 2014; Dolan et al. 2018), but no studies have examined the transporter-releasing capabilities of butylone or pentylone in brain tissue preparations or *in vivo*. To this end, the present study had two major aims: (1) to further explore the molecular mechanism of action for butylone and pentylone using *in vitro* assays, and (2) to determine the *in vivo* neurochemical effects of the drugs after systemic administration in rats.

Materials and Methods

Drugs and Reagents

Chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise specified. Radioactive substrates, [3 H]dopamine, [3 H]5-HT and [3 H] 1-methyl-4-phenylpyridinium ([3 H]), were purchased from PerkinElmer Life Sciences (Waltham, MA, USA). 1-(1,3-Benzodioxol-5-yl)-2-(methylamino)butan-1-one HCl (butylone) and 1-(1,3-benzodioxol-5-yl)-2-(methylamino)pentan-1-one HCl (pentylone) were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Animals and Housing

Male-Sprague-Dawley rats (Envigo, Frederick, MD, USA) weighing 300-400 g were double-housed under conditions of controlled temperature (22 ± 2 °C) and humidity ($45 \pm 5\%$), with food and water freely available. Rats were maintained in facilities accredited by

the Association for the Assessment and Accreditation of Laboratory Animal Care, and procedures were carried out in accordance with the Animal Care and Use Committee of the NIDA IRP. Lights were on from 0700-1900 h and experiments were carried out between 0900-1400 h.

Cell culture

HEK-293 cells stably expressing human DAT or SERT carrying a yellow fluorescent tag or YFP were used for uptake and release studies in cells (Sucic et al. 2010). The tag at the *N*-terminal end of both transporters does not alter their functional characteristics (Schmid et al. 2001). The HEK-293 cells were cultivated on 10 cm dishes in DMEM complemented with fetal calf serum (10%), L-glutamine (1%), glucose (4.5 mg/mL) and gentamycin (50 µg/mL) at 37°C, 95 % humidity and 5 % CO₂.

Uptake assays in rat brain synaptosomes and HEK-293 cells

Uptake inhibition assays in synaptosomes and cells were carried out as previously described (Baumann et al. 2013; Hofmaier et al. 2014; Saha et al. 2015). Briefly, rats were euthanized by CO₂ narcosis, and brains were processed to yield synaptosomes. Rat caudate tissue was used for DAT assays whereas rat forebrain minus caudate was used for SERT assays. Brain tissue was homogenized in ice cold 10% sucrose followed by centrifugation at 1000 *g* for 10 minutes. The supernatant containing crude synaptosomes was kept on ice until use in uptake or release assays.

For uptake inhibition assays in synaptosomes, 5 nM [³H]dopamine or [³H]5-HT was used to assess transport activity at DAT or SERT, respectively. Assays were initiated by adding 100 µL of tissue to 900 µL Krebs-phosphate buffer (126 mM NaCl, 2.4 mM KCl, 0.83 mM CaCl₂, 0.8 mM MgCl₂, 0.5 mM KH₂PO₄, 0.5 mM Na₂SO₄, 11.1 mM glucose, 0.05 mM pargyline, 1 mg/mL bovine serum albumin, and 1 mg/mL ascorbic acid, pH 7.4) containing test drug and [³H]transmitter. Concentrations of butylone or pentylone ranging from 0.1 nM to 10 µM were tested in triplicate. Uptake inhibition assays were terminated by rapid vacuum filtration through Whatman GF/B filters, and retained radioactivity was quantified by liquid scintillation counting.

For the uptake assays in cells, HEK-293 cells were washed twice with Krebs HEPES buffer (10 mM HEPES, 130 mM NaCl, 1.3 mM KH₂PO₄, 1.5 mM CaCl₂, 0.5 mM MgSO₄, pH 7.4). Test compounds were added to cells at concentrations ranging from 0.1 nM to 100 µM for a 5-min preincubation period to allow for equilibration with transporters. Subsequently, [³H]substrates were added, and the reaction was stopped after 1 minute by washing with 500 µL of ice-cold Krebs HEPES buffer. Cells were lysed with 500 µL of 1% sodium dodecyl sulphate and counted by liquid scintillation counting.

Release assays in rat brain synaptosomes and HEK-293 cells

Release assays in synaptosomes and cells were carried out as previously described (Baumann et al. 2013; Hofmaier et al. 2014; Saha et al. 2015). For release assays in synaptosomes, 9 nM [³H]MPP⁺ was used as the radiolabeled substrate for DAT while 5 nM [³H]5-HT was used as a substrate for SERT. All buffers used in the release assay methods

contained 1 μM reserpine to block vesicular uptake of substrates. 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. Concentrations of butylone or pentylone ranging from 0.1 nM to 10 μM were tested in triplicate. Release was terminated by vacuum filtration and retained radioactivity was quantified as described for uptake inhibition.

For release assays in cells, HEK-293 cells were seeded (40,000 cells/well) on glass coverslips (5 mm in diameter) coated with poly-D-lysine. Just prior to the experiment, 0.3 μM of the radiolabeled transporter substrate, [^3H]MPP $^+$, was added to the cells and incubated for 20 min at 37°C. The coverslips were transferred to 0.2 mL chambers and superfused with Krebs Ringer HEPES buffer (10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 20 mM glucose, final pH 7.4) at 25°C, at a rate of 0.7 mL/min. The experiment was started with the collection of fractions every 2 min. The baseline for efflux of radioactivity was established by a washout period of 40 minutes, followed by addition of test compounds alone (10 μM) or test compounds with monensin (10 μM). Monensin is a selective ionophore inducing transmembrane exchange of sodium ions for protons. As such, monensin facilitates dissipation of plasma membrane sodium gradients, allowing sodium entry into the cytosol. The increase in intracellular sodium levels facilitates transporter-mediated reverse transport and promotes substrate efflux. The fractions were collected every 2 min thereafter. The experiment was terminated by lysis of the cells with 1% sodium dodecyl sulphate and counted by liquid scintillation counting.

Measurement of transporter-mediated ionic currents

HEK-293 cells stably expressing human DAT or SERT were seeded at low density 24 h before recording. The currents induced by test compounds were recorded with an Axopatch 200B amplifier under voltage-clamped condition using whole cell configuration. The resistance of the electrode was between 2-4 M Ω . The pipette was filled with internal solution (133 mM K-gluconate, 6 mM NaCl, 1 mM CaCl_2 , 0.7 mM MgCl_2 , 10 mM EGTA, 10 mM HEPES, pH 7.2). All test drugs were dissolved in the external bath solution (140 mM NaCl, 3 mM KCl, 2.5 mM CaCl_2 , 2 mM MgCl_2 , 20 mM glucose, 10 mM HEPES, pH 7.4) and were supplied by a DAD-12 device (Adams & List, Westbury, NY, USA).

The cells were continuously superfused with external solution or the solutions containing different concentrations of butylone or pentylone (300 nM, 1 μM , 10 μM , 30 μM , 100 μM and 300 μM). The holding potential used for recording was -60 mV in all cases. Current amplitudes in response to application of drugs were quantified using Clampfit 10.2 software. Passive holding currents were subtracted, and the traces were filtered using a 100 Hz digital Gaussian low-pass filter.

In vivo neurochemical and behavioral testing

Microdialysis sampling was carried out as described, with minor modifications (Baumann et al. 2012; 2013). Rats were anesthetized with i.p. ketamine (75 mg/kg) and xylazine (5 mg/kg). Once fully anesthetized, each rat received a surgically-implanted jugular catheter and an intracranial guide cannula aimed at the nucleus accumbens (coordinates AP +1.6 mm and

ML –1.7 mm relative to bregma, and –6.2 mm relative to dura). Rats were allowed at least one week for recovery from surgery.

On the evening before an experiment, rats were placed into square Plexiglas arenas (43 cm³) equipped with photobeam arrays to detect motor activity (Truscan, Harvard Apparatus, Holliston, MA, USA) for overnight acclimation. On the next morning, microdialysis probes with 2 × 0.5 mm polyarylethersulfone membranes (CMA/12, Harvard Apparatus) were inserted into the guide cannulas, and extension tubes were attached to jugular catheters. Each rat was connected to a tethering system and back into the arena, and the activity monitoring system was turned on.

Probes were perfused with Ringers' solution pumped at a flow rate of 0.6 µL/min, and dialysate samples were collected at 20-min intervals. Samples were immediately assayed for dopamine and 5-HT by microbore HPLC coupled to electrochemical detection as described elsewhere (Baumann et al. 2011).

Butylone, pentylone or saline was administered to separate groups of rats. Once three stable baseline samples were obtained (<20% variation in dopamine values), rats received two sequential i.v. injections of butylone or pentylone: 1 mg/kg at time zero, followed by 3 mg/kg 60 min later. Control rats received sequential i.v. injections of saline (1 mL/kg) according to the same schedule. Microdialysis samples were collected every 20 min throughout the post-injection period for 120 min. At the end of the experiments, rats were euthanized by CO₂ narcosis and decapitated. Brain sections were examined to verify placement of microdialysis probe tips within the nucleus accumbens. Only those rats with correct placements were included in data analyses.

Data Analysis and Statistics

All data were analyzed using GraphPad Prism software (Prism Version 7, La Jolla, CA, USA). Dose-response results from uptake inhibition and release assays were subjected to nonlinear regression to obtain potency estimates as IC₅₀ and EC₅₀ values, respectively. The effects of monensin on drug-induced efflux were analyzed by two-way ANOVA (treatment × time), followed by Tukey's post hoc tests at individual time points. Drug-induced ionic current measures were analyzed by one-way-ANOVA (dose). *In vivo* microdialysis and locomotor data were analyzed using two-way ANOVA (treatment × time), followed by Bonferroni's post hoc tests at specific time points after drug injection. $p < 0.05$ was considered the minimum criteria for significance.

Results

Butylone and pentylone inhibit uptake at DAT and SERT

Butylone and pentylone were first evaluated in uptake inhibition assays. Figures 2A and 2B show dose-response data from rat brain synaptosomes, whereas Figures 2C and 2D show comparable data from cells transfected with human DAT or SERT. In synaptosomes, butylone and pentylone were fully efficacious blockers at DAT, but pentylone had higher potency for inhibiting [³H]DA uptake (IC₅₀ = 0.12±0.01 µM) compared to butylone (IC₅₀ = 0.40±0.02 µM). Both drugs were weaker at SERT, with IC₅₀ values for inhibiting [³H]5-HT

uptake of 1.43 ± 0.16 and 1.36 ± 0.10 μM for butylone and pentylone, respectively. Results from HEK-293 cells stably expressing DAT or SERT were similar to those from synaptosomes. Butylone and pentylone were efficacious inhibitors at DAT, but pentylone had higher potency ($\text{IC}_{50} = 0.31 \pm 0.07$ μM) than butylone ($\text{IC}_{50} = 1.44 \pm 0.10$ μM). Both drugs were weaker at SERT, with IC_{50} values of 24.4 ± 2.0 and 11.7 ± 0.5 μM for butylone and pentylone. Pentylone was a more potent blocker at DAT than butylone in both assay systems, indicating that increasing the α -carbon chain length of methylone increases selectivity for DAT.

Butylone and pentylone induce efflux at SERT but not DAT

Uptake inhibition assays are useful for identifying drugs which interact at monoamine transporters, but release assays are required to distinguish drugs acting as substrates from those acting as pure uptake blockers. Thus, we next tested the ability of butylone and pentylone to act as substrates and produce transporter-mediated release from synaptosomes and cells. Figures 3A and 3B show dose-response data from rat brain synaptosomes whereas Figures 3C and 3D show data from cells transfected with human DAT or SERT. As depicted in Figure 3 A, butylone and pentylone failed to produce efficacious release of [^3H]MPP $^+$ at DAT. By contrast, Figure 3B shows that both drugs acted as SERT substrates to release [^3H]5-HT, with EC_{50} values of 0.33 ± 0.04 and 1.03 ± 0.18 μM for butylone and pentylone, respectively. It is important to note that the SERT-mediated release evoked by pentylone displayed low efficacy (i.e., 48% of E_{max}), indicating a partial releasing effect.

In HEK-293 cells, we examined the releasing effects of 10 μM butylone or pentylone in the absence or presence of 10 μM monensin. Our previous work showed that monensin is able to selectively enhance efflux produced by transporter substrates, so this compound can be used to distinguish between substrates and blockers (Scholze et al. 2000; Baumann et al. 2013). As shown in Figure 3C, neither butylone nor pentylone evoked [^3H]MPP $^+$ efflux at DAT, with or without monensin. By contrast, both drugs evoked [^3H]5-HT efflux at SERT, and monensin markedly enhanced the effects of butylone ($F_{1,168}=396.6$, $p<0.0001$) and pentylone ($F_{1,144}=202.8$, $p<0.0001$). Tukey's post hoc tests at each time point after butylone or pentylone perfusion revealed that monensin enhanced the efflux produced by butylone more than its effects on pentylone at all time points post-drug treatment. Thus, consistent with the synaptosome findings, butylone tended to increase SERT-mediated [^3H]5-HT release greater than pentylone. Taken together, the release data from synaptosomes and cells suggest that increasing α -carbon chain length of methylone converts DAT substrates to blockers, while substrate activity at SERT remains intact.

Butylone and pentylone induce inward currents at SERT but not DAT

We next wished to examine the ability of butylone and pentylone to produce transporter-mediated ionic currents. Previous work has shown that substrates, but not blockers, are capable of generating transporter-mediated inward sodium currents (Sonders et al. 1997; Sitte et al. 1998; Baumann et al. 2014). HEK-293 cells stably expressing human DAT or SERT were voltage clamped to -60 mV and continuously superfused with bath solution containing different concentrations of drugs to measure their current response. As shown in Figures 4A and 4C, no discernable currents were produced by butylone or pentylone at DAT.

However, both drugs produced dose-related induction of SERT-mediated inward currents, consistent with the activity of transportable substrates (Figures 3B and 3D). It is noteworthy that the magnitude of SERT-mediated current for both drugs was close to that produced by the endogenous substrate 5-HT. Data in Figure 5 show that the dose-response relationship for butylone and pentylone to induce SERT-mediated currents is an inverted U-shaped function. Both butylone and pentylone induced SERT-mediated current and there was no statistical difference between their response. The downward limb of the inverted U-shaped curve is an inhibitory effect of substrates, presumably due to their affinity for the inward conformation of the transporter (Sandtner et al. 2014).

Butylone and pentylone increase extracellular monoamines and motor activity

In the last experiments, we examined effects of intravenous butylone or pentylone on neurochemistry and motor behavior in rats under going *in vivo* microdialysis in the nucleus accumbens. Figures 6A and 6B depict the effects of drugs on dialysate dopamine and 5-HT, whereas Figures 6C and 6D show forward locomotion (i.e., motor activity) and repetitive movements (i.e., stereotypy). Figure 6A demonstrates that drug treatments significantly affected extracellular dopamine when compared to saline control ($F_{2,144} = 9.845$, $p < 0.0001$), with both drugs increasing dopamine to a similar extent. After 1 mg/kg of butylone or pentylone, extracellular dopamine levels were elevated 2-fold above baseline; after 3 mg/kg, dopamine levels increased about 3.5-fold. Figure 6B shows that drug treatments significantly affected extracellular 5-HT as well ($F_{2,144} = 15.76$, $p < 0.0001$), but butylone produced larger increases in 5-HT compared to pentylone ($p < 0.05$, Bonferroni's post hoc test). After 1 mg/kg, butylone increased 5-HT levels 3-fold but pentylone had no effect; after 3 mg/kg, butylone increased 5-HT levels by nearly 7-fold while pentylone afforded a 3-fold rise. The microdialysis results indicate that butylone and pentylone elevate extracellular dopamine to comparable levels but butylone has more robust effects on 5-HT.

Figure 6C demonstrates that both drugs increased motor activity ($F_{2,144} = 15.23$, $p < 0.0001$) but effects of pentylone were substantially greater ($p < 0.05$, Bonferroni's post hoc test). Figure 6D shows that drug treatments affected stereotypy ($F_{2,144} = 38.95$, $p < 0.0001$), while post hoc tests revealed pentylone increased stereotypy and butylone did not. In general, pentylone produced greater stimulation of hyperactivity compared to effects of butylone.

Discussion

The goal of the present study was to characterize the mechanism of action and *in vivo* pharmacological effects of butylone and pentylone, two replacement analogs of methyl one, a synthetic cathinone found in products sold as "bath salts". The Drug Enforcement Administration (DEA) recently placed butylone and pentylone into permanent Schedule I control, along with several other synthetic cathinone analogs (Drug Enforcement Administration 2017). Converging lines of evidence from our *in vitro* studies show that butylone and pentylone display hybrid transporter activity, acting as blockers at DAT but substrates at SERT (see also Eshleman et al. 2013; Simmler et al. 2013; 2014). Data from uptake inhibition assays in synaptosomes and cells agree that pentylone is more potent at

DAT when compared to butylone. Our *in vivo* microdialysis studies reveal for the first time that butylone and pentylone increase extracellular concentrations of dopamine and 5-HT in the brain, consistent with the locomotor activation produced by the drugs (Lopez-Arnau et al. 2012; Gatch et al. 2013; 2015). Importantly, pentylone has predominant effects on extracellular dopamine while butylone has predominant effects of extracellular 5-HT. Our neurochemical results may help to explain the greater reinforcing effects of pentylone when compared to methylone or butylone (Dolan et al. 2018; Javadi-Paydar et al. 2018). Overall, the present findings suggest that effects of drugs on DAT produce elevations in extracellular dopamine and drive locomotor activity, whereas effects on 5-HT may serve to dampen dopamine-mediated actions (Baumann et al. 2011; Schindler et al. 2016).

In rat brain synaptosomes, we found that butylone and pentylone are efficacious inhibitors of DAT and SERT, but pentylone is more DAT-selective. We and others have used the DAT/SERT ratio for a given drug (i.e., $[\text{DAT IC}_{50}]^{-1}/[\text{SERT IC}_{50}]^{-1}$) as an index of transporter selectivity, where higher value indicates greater DAT-selectivity (Baumann et al. 2012; Simmler et al. 2013; Bonano et al. 2015). Here we show that butylone has a DAT/SERT ratio of 3.6 in synaptosome uptake assays, whereas pentylone has a DAT/SERT ratio of 11.4. Lopez-Arnau et al. (2012) examined the effects of butylone on uptake in rat brain synaptosomes and found the drug was more potent at SERT than DAT. In cells transfected with human DAT and SERT, we show that butylone and pentylone are efficacious uptake blockers, and pentylone is somewhat more potent at DAT over SERT. Simmler et al. found similar results in HEK-293 cells and reported that butylone has a DAT/SERT ratio of 2.1 whereas pentylone has a DAT/SERT ratio of 6.2 (Simmler et al. 2013; 2014). Taken together, the uptake inhibition data are consistent with the notion that increasing the α -carbon alkyl chain length of methylone increases potency at DAT relative to SERT.

Uptake inhibition assays are useful for identifying drugs which target monoamine transporters, but release assays are required to discriminate between drugs acting as substrates versus those acting as blockers (Rothman and Baumann 2003; Baumann et al. 2013). Transporter substrates display a number of unique features when compared to transporter blockers, since substrates are translocated along with sodium ions through the transporter permeation pore into the intracellular medium. Secondary to this translocation process, substrates induce inwardly-directed sodium currents and evoke transporter-mediated neurotransmitter efflux (i.e., release) by reversing the normal direction of transporter flux (reviewed in Reith et al. 2015; Sitte and Freissmuth 2015). Using release assays in rat brain synaptosomes, we found that butylone and pentylone do not evoke efflux of $[^3\text{H}]\text{MPP}^+$ at DAT, while both drugs evoke efflux of $[^3\text{H}]\text{-5-HT}$ at SERT. The 5-HT-releasing potency of butylone ($\text{EC}_{50}=0.33 \mu\text{M}$) is greater than that of pentylone ($\text{EC}_{50}=103 \mu\text{M}$). Additionally, the magnitude of SERT-mediated efflux evoked by pentylone reaches only about half of the maximal effect, indicative of so-called “partial release”. The precise underpinnings of partial release are not fully understood but may be related to a slower rate of reverse transport afforded by certain substrates, especially those with increased steric bulk (Rothman et al. 2012).

Our synaptosome release data with pentylone prompted us to examine its substrate activity in cells transfected with pure populations of human DAT or SERT. Prior studies using cells

expressing human transporters show that butylone and pentylone do not evoke release of [^3H]dopamine via DAT but do evoke release of [^3H]5-HT via SERT (Eshleman et al. 2013; Simmler et al. 2013; 2014). Consistent with the findings of others, we show that butylone and pentylone fail to evoke DAT-mediated efflux in transfected cells, whereas both drugs evoke SERT-mediated efflux. Importantly, the SERT-mediated efflux of [^3H]5-HT evoked by butylone and pentylone is markedly enhanced by the ionophore monensin. We have previously shown that monensin selectively enhances the releasing effects of transporter substrates but not blockers (Scholze et al. 2000; Baumann et al. 2013). The monensin experiments provide compelling evidence that butylone and pentylone act as DAT blockers but SERT substrates. Nevertheless, it is noteworthy that the most potent effect of pentylone is DAT blockade, whereas the most potent effect of butylone is SERT-mediated release.

Another powerful method for discriminating transporter substrates from blockers is measurement of their electrophysiological effects at transporters. It is well established that ring-substituted cathinones like mephedrone are transporter substrates that induce inward sodium currents at DAT and SERT (Cameron et al. 2013; Solis 2017). Here we show that pentylone and butylone fail to induce measurable DAT-mediated currents in HEK-293 cells, yet both drugs induce robust SERT-mediated currents. Thus, both drugs display the unique electrophysiological signature of a DAT blocker and a SERT substrate. We have previously shown that the mephedrone analog, 4-methyl-*N*-ethylcathinone (4-MEC), has a similar profile, acting as a DAT blocker and SERT substrate (Saha et al. 2015). In contrast to our work, a recent paper by Dolan et al. (2018) reported that butylone and pentylone induce transporter-mediated currents at both DAT and SERT. However, the magnitude of drug-induced currents at DAT measured by Dolan et al. were only a fraction of the current induced by the endogenous substrate dopamine (i.e., <20%), even when 100 μM concentrations of butylone and pentylone were administered. We have no explanation for the discrepancies between the present electrophysiological findings and those of Dolan et al. (2018), but we present several lines of evidence from rat brain synaptosomes and HEK-293 cells that butylone and pentylone are pure DAT blockers devoid of substrate activity. The majority of findings support the notion that increasing α -carbon chain length of methylone converts DAT substrates to blockers, while maintaining substrate activity at SERT.

The present microdialysis findings are the first demonstration of the *in vivo* neurochemical effects of butylone and pentylone. While both drugs increase extracellular concentrations of dopamine and 5-HT in the nucleus accumbens, butylone has predominant effects on dialysate 5-HT while pentylone has predominant effects on dialysate dopamine. Interestingly, butylone has much weaker locomotor stimulant effects when compared to pentylone, at least at the doses used in our study. These findings support the notion the locomotor activation is related to elevations in extracellular dopamine, and concomitant elevations in extracellular 5-HT can dampen dopamine-mediated stimulant effects (Baumann et al. 2011; Cozzi et al. 2013; Schindler et al. 2016; Suyama et al. 2016). Our neurochemical findings may also provide clues to the reported reinforcing effects of butylone and pentylone in rats (Dolan et al. 2018; Javadi-Paydar et al. 2018). For example, Dolan et al. recently used a rat self-administration paradigm to show that pentylone is a more efficacious reinforcer when compared to butylone or methylone. The same investigators employed drug discrimination assays to show pentylone engenders a stimulus

cue that is more methamphetamine-like whereas butylone is more MDMA-like. Collectively, the behavioral data are consistent with the idea that pentylone is a more dopaminergic drug than butylone, which renders the former more reinforcing than the latter.

In summary, we provide substantial evidence that butylone and pentylone are psychomotor stimulant drugs which display hybrid transporter activity, characterized by blocker effects at DAT but substrate effects at SERT. From a structure-activity perspective, our results demonstrate that increasing the α -carbon alkyl chain length of methylone converts DAT substrates to blockers while substrate activity at SERT is preserved. In brain tissue preparations, the most potent effect of pentylone is DAT blockade while the most potent effect of butylone is SERT-mediated release. The predominant dopaminergic actions of pentylone seem to give this drug greater stimulant and reinforcing properties when compared to butylone, but both drugs are readily self-administered, suggesting both could have substantial abuse liability. Recent forensic data demonstrate that butylone and pentylone are being found in powders, pills and tablets sold as “Molly” or Ecstasy (Palamar et al 2017; Salomone et al. 2017). Unintentional exposure to these drugs or other synthetic cathinones represents a serious public health concern, especially with regard to pentylone, which has greater dopaminergic effects when compared to MDMA.

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Figure 1.
Chemical structures of MDMA, methylone, butylone and pentylone.

Figure 2.

Dose-response effects of butylone and pentytone on [³H]neurotransmitter uptake in rat brain synaptosomes and cells transfected with human transporters. Data from rat brain synaptosomes are depicted in Panel A for [³H]DA uptake by DAT and Panel B for [³H]5-HT uptake by SERT. Data from HEK-293 cells are depicted in Panel C for [³H]DA uptake by human DAT and Panel D for [³H]5-HT uptake by human SERT. Effects of butylone and pentytone are shown as closed circles and open squares, respectively. Data are mean $\pm$ SD expressed as percent of control uptake, for n=3 experiments performed in triplicate.

Figure 3.

Effects of butylone and pentylone on transporter-mediated release in rat brain synaptosomes and cells transfected with human transporters. Data from rat brain synaptosomes are depicted in Panel A for [^3H]MPP $^+$ efflux via DAT and Panel B for [^3H]5-HT efflux via SERT; effects of butylone and pentylone are shown as closed circles and open squares, respectively. Data from HEK-293 cells are depicted in Panel C for [^3H]MPP $^+$ efflux via human DAT and Panel D for [^3H]5-HT efflux via human SERT; effects of 10 μM butylone in the presence of Krebs-Henseleit-Phosphate buffer (KHP) or 10 μM monensin are shown as open or closed circles, whereas effects of 10 μM pentylone in the presence of KHP or 10 μM monensin are shown as open or closed squares. Arrows indicate beginning of butylone or pentylone perfusion. Data are mean $\pm$ SD for $n=3$ experiments performed in triplicate.

Figure 4.

Effects of butylone and pentylone on transporter-mediated currents measured in HEK-293 cells stably transfected with human transporters. Representative traces of currents produced by butylone at DAT and SERT are depicted in Panels A and B, respectively. Representative traces of currents produced by pentylone at DAT and SERT are depicted in Panels C and D, respectively.

Figure 5.

Dose-response effects of butylone and pentylone to produce transporter-mediated currents at human DAT and SERT. The Y-axis represents normalized current obtained by dividing current obtained with drug treatment by the current obtained with a 10 μ M concentration of the endogenous substrate. The X-axis represents drug concentration in log scale. Effects of butylone on DAT and SERT are represented by open and closed circles, whereas effects of pentylone on DAT and SERT are represented as open and closed squares. Data are mean $\pm$ SD for n=3 experiments.

Figure 6.

Neurochemical and behavioral effects produced by butylone and pentylone in conscious rats undergoing microdialysis in the nucleus accumbens. Effects of drugs on extracellular dopamine (DA) are shown in Panel A while effects on extracellular 5-HT are shown in Panel B. Effects of drugs on forward locomotion (Motor) and repetitive movements (Stereotypy) are shown in Panels C and D. Arrows indicate time of intravenous injection: 1 mg/kg of butylone or pentylone was given at time zero followed by 3 mg/kg given 60 min later. Saline was injected at 1 mL/kg at the same time points. Data are mean $\pm$ SEM expressed as a percent of basal preinjection values for N=6-7 rats/group. Filled symbols represent significant effects of drugs compared to saline treatment at specific time points, whereas asterisks represent significant differences compared to butylone at specific time points ($p < 0.05$, Bonferroni's test).