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"Polytox" synthetic cathinone abuse: A potential role for organic cation transporter 3 in combined cathinone-induced efflux

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ABSTRACT

Synthetic cathinone derivatives are a new class of psychoactive substances (NPS), also known as "bath salts", designed to exert psychostimulant effects resembling those of well-known psychostimulants, such as cocaine and 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"). As major constituents of bath salts, the cathinone derivatives 3,4-methylenedioxypyrovalerone (MDPV) and 4-methylmethcathinone (mephedrone), have received considerable media attention. MDPV and mephedrone interfere with the function of the high affinity transporters for dopamine (DAT), norepinephrine (NET) and serotonin (SERT), resulting in increased extracellular levels of these monoamines, though their mechanism of action differs. MDPV acts as a non-transported inhibitor of DAT, NET and SERT, whereas mephedrone promotes transporter-mediated release in an amphetamine-like fashion. MDPV and mephedrone are often taken together, creating a conundrum in as much as non-transported inhibitors, like MDPV, prevent mephedrone-induced reverse transport via DAT, NET and SERT. Here we provide evidence supporting a role for organic cation transporter 3 (OCT3) in the actions of mephedrone, which may account for its ability to enhance effects of MDPV. We show that mephedrone can induce substrate efflux via OCT3 in the presence of MDPV. Real-time recordings of the fluorescent OCT3 substrate (4-(4-dimethylamino) styryl)-N-methylpyridinium (ASP^+) and radiotracer-flux studies using [^{3}H]1-methyl-4-phenyl-pyridinium (MPP^+), demonstrated that OCT3 is MDPV-insensitive when expressed in human embryonic kidney (HEK293) cells. *Ex vivo* experiments performed in cultured superior cervical ganglia (SCG) cells, rich in NET and OCT3, revealed that mephedrone induces [^{3}H]MPP^+ release in an OCT3-dependent manner when NET is fully occupied with MDPV. These results extend our recent findings that OCT3 is key in the mechanism of action of amphetamine-induced substrate release. OCT3 likewise appears to be a mechanism through which mephedrone can induce release of monoamines, thereby accounting for the paradoxically more potent psychostimulant effects of MDPV taken together with mephedrone, and greater risk for deleterious side effects.

1. Introduction

Non-medical consumption of psychostimulants is a global phenomenon that places tremendous demands on fiscal and public health care systems (Degenhardt and Hall, 2012). In addition to "classical" psychostimulants, like *S*(+)amphetamine ("speed") and cocaine, the markets are now flooded with novel designer drugs that mimic the action of these "classical" scheduled substances (Baumann and Volkow, 2016). These new psychoactive substances (NPS) are easy to obtain, often distributed via the Internet, and provide powerful, yet (in many cases) legal alternatives to regulated cannabinoids, hallucinogens, opioids and stimulants (Meyer, 2016). NPS are introduced into the markets at an alarming rate. For instance, 251 NPS were reported in 2012, outnumbering the controlled substances that year (i.e. 234

Abbreviations: D22, decynium-22; DAT, dopamine transporter; MAT, monoamine transporter; MDPV, 3,4-methylenedioxypyrovalerone; MEPH, 4-methyl-methcathinone; NET, norepinephrine transporter; OCT, organic cation transporter; SCG, superior cervical ganglion; SERT, serotonin transporter

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scheduled substances; World Drug Report, 2013[2]). In 2014, stimulant-type drugs classified as synthetic cathinones and phenethylamines were the dominant NPS in the European Union (Tyrkko et al., 2016), providing indirect evidence for the widespread abuse of psychostimulants.

Around 2010, NPS derived from the naturally occurring parental compound cathinone were marketed as legal replacements for the stimulants MDMA and cocaine. Mephedrone and MDPV represent two major cathinone derivatives found in "bath salts" or "plant food" products (Ross et al., 2011; Spiller et al., 2011). Deleterious side effects were associated with "bath salt" intoxications (Lusthof et al., 2011; Maskell et al., 2011; Murray et al., 2012; Spiller et al., 2011) and attracted media attention. Mephedrone and MDPV were classified as schedule I substances in 2011 (DEA, 2011). However, these drugs continue to be abused in spite of the their illegal status (Styszko et al., 2016; Hockenhull et al., 2016; Archer et al., 2013, 2014). Stimulant-type "bath salts" elevate the extracellular concentration of monoamines. This is achieved by their ability to disrupt uptake mechanisms for dopamine, norepinephrine and serotonin. Under physiological conditions, the high-affinity transporters for dopamine (i.e. dopamine transporter, DAT), serotonin (i.e. serotonin transporter, SERT) and norepinephrine (norepinephrine transporter, NET) sequester their respective substrates via a secondary-active transport process (Kristensen et al., 2011) and operate in an orchestrated manner with vesicular monoamine transporters (Egana et al., 2009) to replenish vesicular monoamine stores (Sitte and Freissmuth, 2015). Earlier studies conclusively showed that mephedrone acts as a substrate-type releaser, promoting carrier-mediated reverse transport of monoamines via DAT, NET and SERT. On the contrary, the same laboratories showed that MDPV acts as a potent, non-transported inhibitor of DAT and NET, but was a less potent inhibitor of SERT (Baumann et al., 2012; Baumann et al., 2013; Simmler et al., 2013). Interestingly, non-transported inhibitors interfere with the effects of substrate-type releasers. For instance, in the presence of cocaine, *S*(+)-amphetamine fails to induce both inwardly directed currents via DAT stably expressed in HEK293-EM4 cells (Erreger et al., 2008) and NET-mediated release from HEK293-cells stably expressing this transporter (Mayer et al., 2018). Based on its pharmacological profile, these observations indicate that MDPV would counteract substrate-induced reverse transport in a cocaine-like fashion. It is paradoxical then, that co-existence of MDPV and amphetamine-like releasers has been detected in biological samples of a plethora of drug users (Kriikku et al., 2011) and in drug samples that were analyzed by mass-spectrometry by the Viennese drug prevention project "CheckiT ! Check your drugs"[3]).

Recently, we reported that organic cation transporter 3 (OCT3) plays an unsuspected role in the actions of *S*(+)amphetamine (Mayer et al., 2018). OCT3 is a promiscuous low-affinity/high-capacity transporter that has been implicated in monoamine-homeostasis (Daws, 2009; Courousse and Gautron, 2015). OCT3 is cocaine-insensitive (Amphoux et al., 2006), is expressed in neurons (Vialou et al., 2008; Gasser et al., 2017; Mayer et al., 2018; Kristufek et al., 2002), and capable of bidirectional monoamine transport (Koepsell et al., 2007). We found that amphetamine promotes non-exocytotic release of substrates in the presence of cocaine in an OCT3-dependent manner (Mayer et al., 2018). Here we investigated a role for OCT3 in the mixed consumption of MDPV with mephedrone. Our results corroborate a potential contribution of OCT3 to the synergistic effects of MDPV and mephedrone when used in combination.

2. Materials and methods

2.1. Radiotracer-flux experiments

Uptake experiments using [^{3}H]substrates of monoamine transporters were performed as described previously (Mayer et al., 2017). Briefly, the murine (m) or human (h) isoforms of OCT3, DAT, NET and SERT were expressed in human embryonic kidney (HEK293) cells. For uptake experiments, cells expressing the transporter of interest were seeded onto poly-D-lysine (PDL) coated 96-well plates (40,000 cells per well) 24 h prior to the experiments. At the beginning of an experiment, cell culture medium was removed and replaced with pre-warmed krebs-HEPES buffer (KHB, 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM D-glucose, pH 7.3). Subsequently, the cells were pre-incubated with increasing concentrations of test drug for 5 min before [^{3}H]substrate (20 nM [^{3}H]1-methyl-4-phenyl-pyridinium (MPP^+) for mOCT3, hDAT and hNET; 100 nM [^{3}H]5-hydroxytryptamine (5-HT) for hSERT were added. Uptake was terminated after 180 (mOCT3, hDAT, hNET) or 60 s (hSERT) by aspirating the tritiated substrate and washing the cells with ice-cold KHB. Finally, the cells were lysed in 1% sodium-dodecyl sulfate (SDS) and uptake of [^{3}H]substrate was determined by scintillation counting. Non-specific uptake was determined in presence of 10 μM paroxetine (hSERT), 10 μM mazindol (hDAT and hNET) or 10 μM decynium-22 (D22, mOCT3).

Transporter release experiments in superior cervical ganglia (SCGs) were performed as described in (Mayer et al., 2018). SCG cultures were obtained following established protocols (Kristufek et al., 2002; Salzer et al., 2014). For release assays, SCGs were pre-loaded with [^{3}H]MPP^+ (0.1 μM in KHB, 30 min at 37 °C). Subsequently, SCGs were placed into superfusion chambers (volume of 200 μL) and superfused with KHB (flow rate: 0.7 mL per minute) for 40 min to establish a stable baseline before the collection of 2-min fractions was initiated. After three basal fractions, SCGs were superfused with D22 (0.1, 1 and 10 μM) or vehicle for three fractions before MDPV (20 μM) was added to the buffer for another three fractions. Finally, SCGs were exposed to mephedrone (10 μM, five fractions) and then lysed in 1% SDS to determine the total radioactivity. Radioactivity in the superfusates was determined by scintillation counting and the release of [^{3}H]MPP^+ was expressed as fractional rate, i.e. radioactivity released per 2-min fraction is given as the percentage of total radioactivity present at the beginning of each fraction (Sitte et al., 2001). To measure release of [^{3}H]MPP^+ from HEK293 cells co-expressing NET and OCT3, the experiments were performed as described above for SCGs and as described in detail in (Sitte et al., 2001; Mayer et al., 2017).

2.2. Real-time measurements of fluorescent substrate accumulation and release

As described in detail in an earlier publication (Mayer et al., 2018), OCT3-expressing HEK293 cells were grown on CELLVIEW™ (Greiner Bio-one, Kremsmuenster, Austria) glass bottom dishes and superfused with KHB at a constant flow-rate of 2.5 mL per minute, 25 °C). The fluorescent OCT3-substrate (4-(dimethylamino)styryl)-N-methylpyridinium) (ASP^+) and the substances of interest were directly applied to the cells by a microsuperfusion system (Octaflow™, ALA Scientific Instruments Inc., Farmingdale, NY, USA). A Pulsed high-power bluewhite LED (set to 480 nm) (CREE Inc., Racine, WI, USA) was used to excite ASP^+. ASP^+ emission was recorded at 609 nm with a high sensitivity photomultiplier tube photo-detector system (HAMAMATSU Photonics, Hersching am Ammersee, Germany) of a reconfigured Sequoia-Turner 450 fluorometer mounted to an inverted epifluorescence microscope (Olympus IX50, Olympus, Tokyo, Japan). OCT3 expressing cells were superfused with KHB for 20 s before ASP^+ (3 μM) was applied to the cells for 40 s. Subsequently, the cells were superfused with ASP^+ (3 μM) and the substance of interest for 140 s. The background fluorescence (i.e. the fluorescence at t = 20 s) was subtracted from the entire trace. Analysis was performed using Axon pClamp10.3, Microsoft excel 2010 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

[2] https://www.unodc.org/unodc/secured/wdr/wdr2013/World_Drug_Report_2013.pdf.

[3] https://checkit.wien, accessed in July 2018.

2.3. Drugs and chemicals

[^{3}H]MPP$^+$ was obtained from ARC (St. Louis, MO, USA; specific activity: 80 Ci/mmol), mephedrone was from TOCRIS (Abingdon, United Kingdom) and MDPV was synthesized in its racemic form in the labratory of Nicholas V. Cozzi as described in (Baumann et al., 2013). All other chemical used were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.4. Ethics declaration

Rats were sacrificed by decapitation after short CO_2 asphyxia in accordance with the ARRIVE guidelines and the Austrian animal protection law (see http://www.ris.bka.gv.at/Dokumente/BgblAuth/BGBLA_2012_I_114/BGBLA_2012_I_114.pdf) and the Austrian animal experiment by-laws (see http://www.ris.bka.gv.at/Dokumente/BgblAuth/BGBLA_2012_II_522/BGBLA_2012_II_522.pdf) which implement European (DIRECTIVE, 2010/63/EU; see http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF) in Austrian law.

2.5. Data analysis and statistics

Release of [^{3}H]MPP$^+$ from SCGs was evaluated by two-way analysis of variance (drug treatment x time) followed by Bonferroni's *post hoc* test. Kruskal-Wallis, followed by Dunn's *post hoc* test, was used to analyze uptake of ASP$^+$. $P < 0.05$ was set as the minimum criterion for statistical significance.

3. Results

3.1. Mephedrone and MDPV inhibit DAT, NET and SERT-mediated substrate uptake but not that of OCT3

We first examined the ability of mephedrone and MDPV to inhibit uptake of substrates via OCT3. Fig. 1 shows that neither mephedrone nor MDPV are potent inhibitors of inwardly directed transport of [^{3}H]MPP$^+$ via OCT3. Mephedrone has been reported to be a transported substrate for DAT, NET and SERT (Baumann et al., 2013; Simmler et al., 2013; Mayer et al., 2016). Given the promiscuity of OCT3, substrates of DAT, NET and SERT may also serve as substrates of OCT3 (Koepsell et al., 2007). We therefore investigated the effects of mephedrone on OCT3 in more detail. OCT3-expressing cells were superfused with the fluorescent substrate ASP$^+$ to assess OCT3 mediated-transport in real-time. Fig. 2 illustrates that ASP$^+$ time-dependently accumulates in OCT3 expressing cells. Application of mephedrone (20 and 40 µM) had no direct effect on substrate accumulation in this assay. On the contrary, it is well documented that the OCT3-substrate MPP$^+$ and corticosterone inhibit uptake of ASP$^+$ via OCT3 (Massmann et al., 2014; Mayer et al., 2018). These data suggest that mephedrone does not act as either a competitive or non-competitive inhibitor at OCT3 up to 50 µM. In contrast to its lack of activity at OCT3, mephedrone inhibited uptake mediated by DAT, NET and SERT with equal potency in the low µmolar range (Fig. 3). As expected, MDPV powerfully inhibited DAT and NET, with significantly weaker effects at SERT (Fig. 3).

3.2. Mephedrone induces substrate efflux in the presence of MDPV in an OCT3-dependent manner in SCGs

The observation that OCT3 is MDPV-insensitive prompted us to speculate that combinations of MDPV and mephedrone may affect two different classes of monoamine transporters: i) high-affinity transporters of the SLC6-family, i.e. DAT, NET (and SERT) and ii) OCT3. We used SCGs as a model system, since these neurons richly express NET and OCT3 (Kristufek et al., 2002). SCGs were pre-loaded with [^{3}H]MPP$^+$ and superfused with mephedrone (10 µM) in the presence of MDPV (20 µM). Prior to the addition of MDPV and mephedrone, SCGs were superfused with the OCT3-inhibitor D22 or vehicle. In the absence of D22, mephedrone evoked release of [^{3}H]MPP$^+$, an effect that was lost with increasing concentrations of D22 (Fig. 4). Two-way ANOVA revealed a significant effect of D22 application (drug treatment x time) on [^{3}H]MPP$^+$ efflux from SCGs ($F_{3,\ 39} = 41.13$; $P < 0.0001$). Remarkably, we observed that the addition of MDPV elevated the basal release of tritiated MPP$^+$. To examine this phenomenon in an additional experimental system, we transfected HEK293 cells with NET and OCT3. The direction of OCT3-mediated transport is determined by the electrochemical gradient of its substrates (Koepsell et al., 2007). HEK293 cells co-expressing OCT3 and NET were pre-loaded with [^{3}H]MPP$^+$. As with SCGs, these cells were superfused with cocaine (20 µM) and the OCT3-inhibitor corticosterone (30 µM). When corticosterone was given first, we observed a decrease in basal release of [^{3}H]MPP$^+$. On the contrary, when cocaine was given first, we detected a marked increase in released [^{3}H]MPP$^+$ (Fig. 5). These findings suggest that there is an ongoing "leak" of intracellular substrate, which can most parsimoniously be explained by the presence of OCT3. Simple diffusion of [^{3}H]MPP$^+$ across the plasma membrane is not possible due to its permanent charge (Scholze et al., 2000). In addition, this "leak" is further augmented in SCGs by mephedrone (Fig. 4).

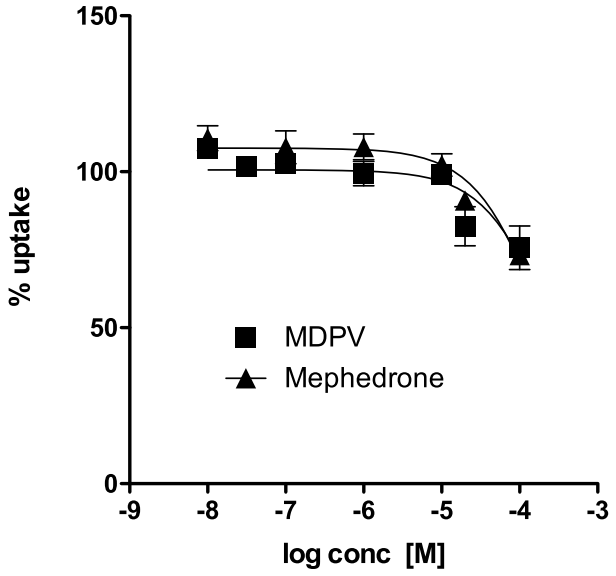

Fig. 1. Effects of mephedrone and MDPV on OCT3-mediated uptake of [^{3}H]MPP$^+$.
HEK293 cells expressing mOCT3 were exposed to increasing concentrations of MPDV and mephedrone, respectively. Data are mean and SEM, n = 3 independent experiments, performed in triplicate.

4. Discussion

The current study investigated potential contributions of OCT3 to the actions of the mixed abuse of MDPV and mephedrone, two of the most popular synthetic cathinones. Earlier work by Cameron and colleagues (Cameron et al., 2013), suggested that the combination of MDPV and mephedrone acts synergistically at monoamine transporters of the SLC6-family: first, mephedrone would induce reverse transport of dopamine via DAT. Subsequently, MDPV would prevent DAT-mediated re-uptake of previously released dopamine. However, at steady state, it seems plausible that MDPV would prevent mephedrone-induced reverse transport of dopamine by blocking its escape through DAT. Consistent with this notion, we observed that amphetamine-induced release of substrate via NET in hNET expressing HEK293 cells was effectively blocked in the presence of cocaine (Mayer et al., 2018). Considering the high affinity of MDPV for DAT and NET (low nanomolar range) (see

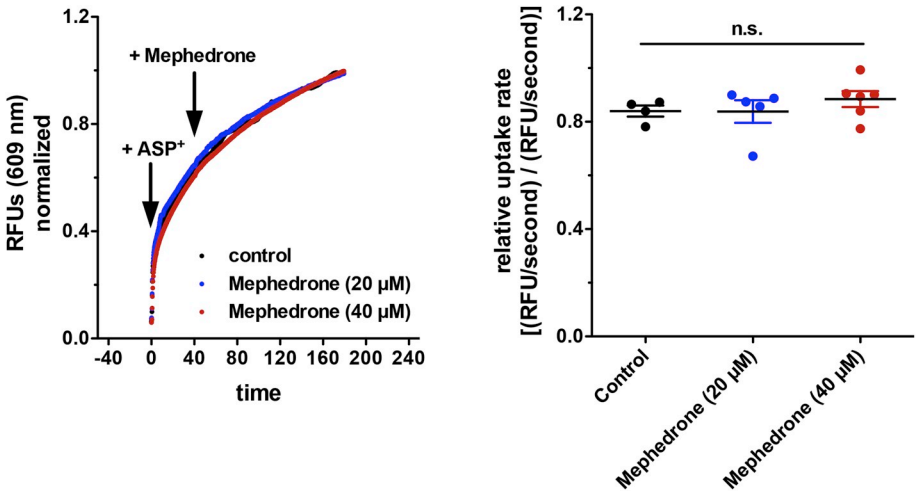

Fig. 2. Effect of mephedrone on OCT3-mediated uptake of ASP^+ in HEK293 cells.
Left panel: hOCT3 expressing HEK293 cells were superfused with ASP^+ (3 µM) for 40 s before mephedrone (20 and 40 µM) were added to the superfusion system.
Right panel: the uptake rates ten seconds after mephedrone application were set in relation to the uptake rates ten seconds before mephedrone was added. N = 4–5 measurements per condition. Data are shown as mean and SEM. Kruskal-Wallis, followed by Dunn's. (n.s. = not significant).

Fig. 3 and (Baumann et al., 2013; Simmler et al., 2013)), it appears unlikely that mephedrone would induce carrier-mediated reverse transport of monoamines via DAT and NET in the presence of MDPV (given the assumption that both drugs are present within a similar concentration range). Remarkably, however, we found that MDPV did not fully block mephedrone-induced [^{3}H]MPP^+ release in SCGs, which express both NET and OCT3.

OCT3 transports various endogenous and exogenous compounds, including monoamines. OCT3 is expressed throughout brain (Gasser et al., 2009), including limbic regions (Gasser et al., 2009) and is expressed in neurons as well as in glial cells (Wu et al., 1998; Kristufek et al., 2002; Amphoux et al., 2006; Gasser et al., 2006; Vialou et al., 2008; Mayer et al., 2018). In contrast to DAT, NET and SERT, OCT3 is largely MDPV- and mephedrone-insensitive (Fig. 1). We found that mephedrone was still able to evoke release of pre-loaded [^{3}H]MPP^+ from OCT3-and NET-rich SCGs in the presence of MDPV applied at a concentration to fully block NET (i.e. more than 300 times higher than its K_i value for NET). Co-incubation of SCGs with the OCT3 inhibitor, D22, concentration-dependently inhibited mephedrone-induced release of [^{3}H]MPP^+ with the highest concentration (10 µM; i.e. 100 times higher than the K_i value of D22 reported for human OCT3 (Hayer-Zillgen et al., 2002)) fully blocking the MDPV-insensitive component of mephedrone-induced [^{3}H]MPP^+ release.

Surprisingly, addition of MDPV resulted in a slight, yet statistically significant increase in the amount of [^{3}H]MPP^+ in the superfusates in the absence of the OCT3 inhibitor D22 (see also Fig. 4). This effect is difficult to reconcile with the molecular mechanism of action of MDPV, i.e. non-transported inhibitor (Baumann et al., 2013; Simmler et al., 2013), however, it is important to note that this result replicates our previous findings (Baumann et al., 2013). Furthermore, low concentrations of MDPV were associated with DAT-mediated release (Shekar et al., 2017); however, non-transported inhibitors are not expected to elevate basal release of tritiated substrates (Scholze et al., 2001). Due to the permanent charge carried by MPP^+, passive diffusion across cellular membranes precludes so-called "pseudo-efflux" events as an explanation (i.e. loss of substrate from the intracellular compartment that is unmasked when ongoing re-uptake is blocked by the presence of inhibitors (Scholze et al., 2000)).

In efforts to understand this apparent anomaly, we co-transfected HEK293 cells with NET and OCT3. Consistent with our finding of increased [^{3}H]MPP^+ in superfusates following MDPV in SCGs (Fig. 4), inhibition of NET with cocaine elevated basal release of [^{3}H]MPP^+. In contrast, [^{3}H]MPP^+ in superfusates was decreased when the cells were challenged with the OCT3-inhibitor corticosterone (Fig. 5). Together, these phenomena suggest that there is a continuous "leak" of substrates via OCT3 under basal conditions and concomitant re-uptake by NET, establishing a balance of extracellular MPP^+. Hence, whenever the "leakage" via OCT3 is reduced by corticosterone, the released concentration of tritiated substrate is lowered because NETs allow for efficient clearance. Upon subsequent addition of cocaine, uptake via NET is inhibited and the concentration of tritiated substrate rises again. Conversely, if NET is blocked first by administration of cocaine,

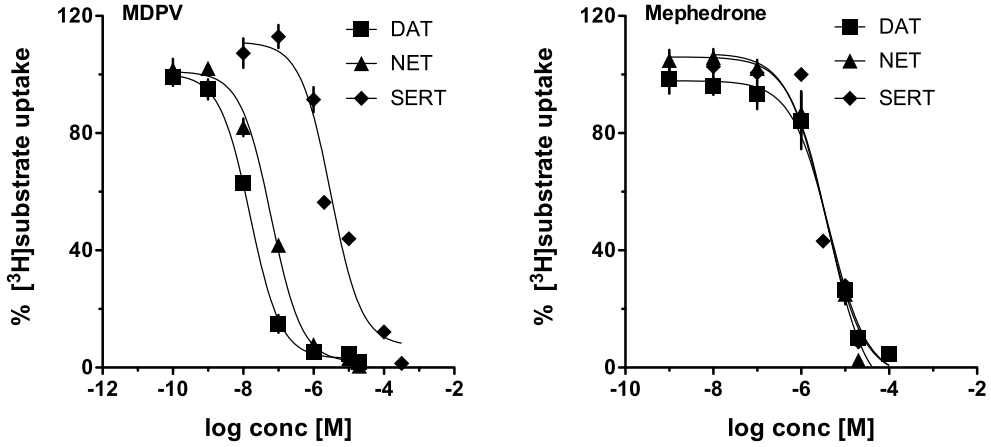

Fig. 3. Effects of mephedrone and MDPV on DAT, NET and SERT mediated uptake.
HEK293-cells expressing the murine isoforms of DAT, NET and SERT were exposed to increasing concentrations of mephedrone or MDPV. Both drugs served as fully efficacious inhibitors of [^{3}H]substrate uptake via each transporter.

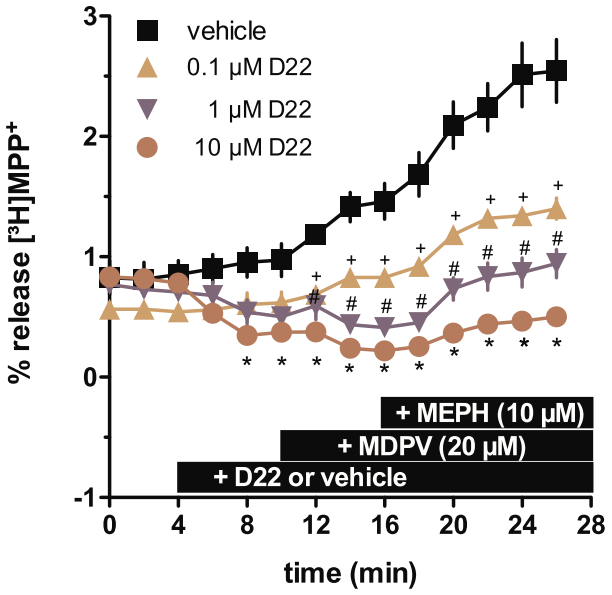

Fig. 4. Mephedrone evoked release of [^{3}H]MPP$^+$ from SCGs.
SCGs were pre-loaded with tritiated MPP$^+$ and superfused with buffer. After three basal fractions, the OCT3-inhibitor (orange triangle up, 0.1 µM; purple triangle down, 1 µM; red circles, 10 µM) or vehicle (black squares) were added before MDPV (20 µM) was added to the superfusion system. Finally, SCGs were exposed to mephedrone (10 µM) which elevated the fractional release rates of [^{3}H]MPP$^+$. N = 9 independent observations per condition. *, + and # indicate P < 0.05 versus MDPV only (Bonferroni's).

extracellular MPP$^+$ rises because NET cannot take up its substrate. Addition of corticosterone then lowers MPP$^+$ extracellularly, because MPP$^+$ cannot leave the cells via OCT3 anymore.

Mephedrone had no direct effects on OCT3, yet affected reverse transport of [^{3}H]MPP$^+$ in SCGs. Disturbance of the vesicular monoamine transporter (VMAT) has been suggested to contribute to the effects of amphetamines. The resulting elevation in cytosolic monoamines renders them available for reverse-transport (Sulzer et al., 2005). Although mephedrone only weakly interacts with VMAT2 (Pifl et al., 2015), it remains theoretically possible that mephedrone elevates the cytosolic concentration of the VMAT2-substrate [^{3}H]MPP$^+$ which then, following its electrochemical gradient, leaks out via OCT3. Our findings provide further evidence for OCT3 as an important player in the ability of amphetamine and amphetamine-like psychostimulants to promote non-exocytotic release of monoamines. It is worth mentioning that releasers also inhibit uptake mediated by DAT, NET and SERT. This phenomenon simply results from the fact that releasers act as substrates, hence as competitive inhibitors of their respective transporters. However, as demonstrated earlier, reverse-transport rather than inhibition of inwardly-directed transport appears to be the primary mechanism driving the effects of amphetamines *in vivo* (Liechti et al., 2000).

Given that psychostimulants are increasingly used as mixtures, consisting of cocaine- and amphetamine-like drugs, OCT3 may serve as an explanation - in addition to the effects of these combinations on the pharmacokinetics of the abused drugs (Benturquia et al., 2018) - for the ability of these "combos" to produce a greater "high", as well as greater risk for deleterious effects associated with psychostimulant abuse.

5. Conclusion

The data at hand indicate that the combination of MDPV and mephedrone targets high-affinity and low-affinity monoamine transporters, which may underlie the synergistic effects of this combination. Mephedrone promotes carrier-mediated reverse transport via MDPV-insensitive OCT3. Simultaneously, MDPV blocks the high-affinity transporters for dopamine and norepinephrine (and to a lesser extent the serotonin transporter). Therefore, by interrupting the function of DAT, NET and SERT and by promoting release via OCT3, this drug combination may result in drastically elevated extracellular monoamine concentrations and may lead to deleterious side effects.

Competing interests

H.H.S. has received honoraria for lectures and consulting from AbbVie, Aesca, Amgen, Astellas, Astra Zeneca, Astropharma, Gebro, IMH, IIR, Janssen-Cilag, Lundbeck, MSD, Mundipharma, Pfizer, Ratiopharm, Roche, Sandoz, Sanofi-Aventis, Serumwerk Bernburg, and Shire (past 5 years). All other authors declare no competing financial interests.

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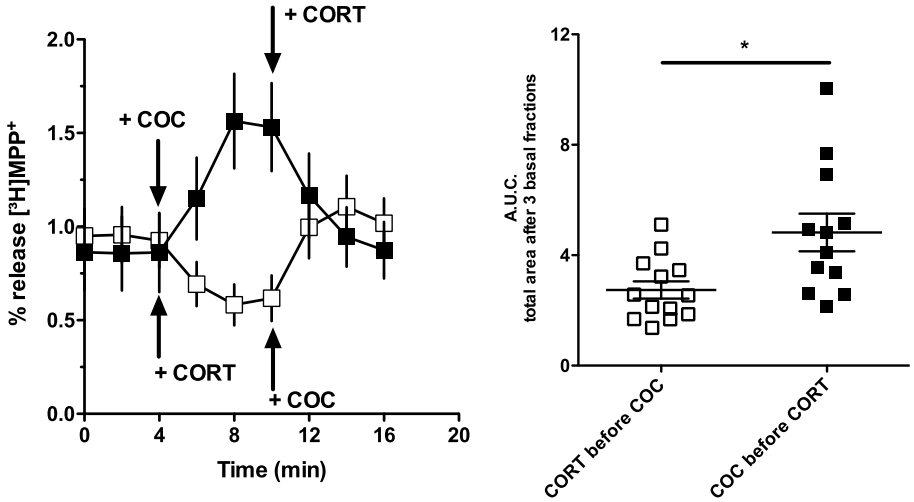

Fig. 5. Effects of cocaine and corticosterone on the efflux rates of tritiated substrate from NET and OCT3 co-expressing cells.
HEK293 cells expressing hNET and mOCT3 were preloaded with [^{3}H]MPP$^+$ and superfused with buffer. After three basal fractions, cocaine (20 µM) or corticosterone (30 µM) were added to the superfusion system. After three additional fractions, corticosterone or cocaine was added to ensure the combined treatment with both drugs in a sequential order. Data are shown as mean and SEM for n = 13 (CORT before COC) and n = 12 (COC before CORT) independent observations. The area under curve was calculated for each individual trace and plotted on the right-hand panel. Data were analyzed by unpaired *t*-test (two-tailed) with Welch's correction (P = 0.0141).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.09.008.

References

Amphoux, A., Vialou, V., Drescher, E., Bruss, M., Mannoury la Cour, C., Rochat, C., Millan, M.J., Giros, B., Bonisch, H., Gautron, S., 2006. Differential pharmacological in vitro properties of organic cation transporters and regional distribution in rat brain. Neuropharmacology 50, 941–952.

Archer, J.R., Dargan, P.I., Hudson, S., Wood, D.M., 2013. Analysis of anonymous pooled urine from portable urinals in central London confirms the significant use of novel psychoactive substances. QJM 106, 147–152.

Archer, J.R., Dargan, P.I., Lee, H.M., Hudson, S., Wood, D.M., 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. 52, 160–165.

Baumann, M.H., Volkow, N.D., 2016. Abuse of new psychoactive substances: threats and solutions. Neuropsychopharmacology 41, 663–665.

Baumann, M.H., Ayestas JR., M.A.,, Partilla, J.S., Sink, J.R., Shulgin, A.T., Daley, P.F., Brandt, S.D., Rothman, R.B., Ruoho, A.E., Cozzi, N.V., 2012. The designer methcathinone analogs, mephedrone and methylone, are substrates for monoamine transporters in brain tissue. Neuropsychopharmacology 37, 1192–1203.

Baumann, M.H., Partilla, J.S., Lehner, K.R., Thorndike, E.B., Hoffman, A.F., Holy, M., Rothman, R.B., Goldberg, S.R., Lupica, C.R., Sitte, H.H., Brandt, S.D., Tella, S.R., Cozzi, N.V., Schindler, C.W., 2013. Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive 'bath salts' products. Neuropsychopharmacology 38, 552–562.

Benturquia, N., Chevillard, L., Poire, C., Roussel, O., Cohier, C., Decleves, X., Laplanche, J.L., Etheve-Quelquejeu, M., Chen, H., Megarbane, B., 2018 Jul 3. Is the 3,4-methylendioxypyrovalerone/mephedrone combination responsible for enhanced stimulant effects? A rat study with investigation of the effect/concentration relationships. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-018-4962-0. [Epub ahead of print] PubMed PMID: 29971461.

Cameron, K.N., Kolanos, R., Solis JR., E.,, Glennon, R.A., De Felice, L.J., 2013. Bath salts components mephedrone and methylenedioxypyrovalerone (MDPV) act synergistically at the human dopamine transporter. Br. J. Pharmacol. 168, 1750–1757.

Courousse, T., Gautron, S., 2015. Role of organic cation transporters (OCTs) in the brain. Pharmacol. Ther. 146, 94–103.

Daws, L.C., 2009. Unfaithful neurotransmitter transporters: focus on serotonin uptake and implications for antidepressant efficacy. Pharmacol. Ther. 121, 89–99.

Drug Enforcement Administration, 2011. Schedules of controlled substances: temporary placement of three synthetic cathinones in Schedule I. Final Order. In: 2011/10/25 edn. Fed Regist Vol. 76. pp. 65371–65375.

Degenhardt, L., Hall, W., 2012. Extent of illicit drug use and dependence, and their contribution to the global burden of disease. Lancet 379, 55–70.

Egana, L.A., Cuevas, R.A., Baust, T.B., Parra, L.A., Leak, R.K., Hochendoner, S., Pena, K., Quiroz, M., Hong, W.C., Dorostkar, M.M., Janz, R., Sitte, H.H., Torres, G.E., 2009. Physical and functional interaction between the dopamine transporter and the synaptic vesicle protein synaptogyrin-3. J. Neurosci. 29, 4592–4604.

Erreger, K., Grewer, C., Javitch, J.A., Galli, A., 2008. Currents in response to rapid concentration jumps of amphetamine uncover novel aspects of human dopamine transporter function. J. Neurosci. 28, 976–989.

Gasser, P.J., Lowry, C.A., Orchinik, M., 2006. Corticosterone-sensitive monoamine transport in the rat dorsomedial hypothalamus: potential role for organic cation transporter 3 in stress-induced modulation of monoaminergic neurotransmission. J. Neurosci. 26, 8758–8766.

Gasser, P.J., Orchinik, M., Raju, I., Lowry, C.A., 2009. Distribution of organic cation transporter 3, a corticosterone-sensitive monoamine transporter, in the rat brain. J. Comp. Neurol. 512, 529–555.

Gasser, P.J., Hurley, M.M., Chan, J., Pickel, V.M., 2017 May. Organic cation transporter 3 (OCT3) is localized to intracellular and surface membranes in select glial and neuronal cells within the basolateral amygdaloid complex of both rats and mice. Brain Struct. Funct. 222 (4), 1913–1928. https://doi.org/10.1007/s00429-016-1315-9. Epub 2016 Sep 22.

Hayer-Zillgen, M., Bruss, M., Bonisch, H., 2002. Expression and pharmacological profile of the human organic cation transporters hOCT1, hOCT2 and hOCT3. Br. J. Pharmacol. 136, 829–836.

Hockenhull, J., Murphy, K.G., Paterson, S., 2016. Mephedrone use is increasing in London. Lancet 387, 1719–1720.

Koepsell, H., Lips, K., Volk, C., 2007. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm. Res. (N. Y.) 24, 1227–1251.

Kriikku, P., Wilhelm, L., Schwarz, O., Rintatalo, J., 2011. New designer drug of abuse: 3,4-Methylenedioxypyrovalerone (MDPV). Findings from apprehended drivers in Finland. Forensic Sci. Int. 210, 195–200.

Kristensen, A.S., Andersen, J., Jorgensen, T.N., Sorensen, L., Eriksen, J., Loland, C.J., Stromgaard, K., Gether, U., 2011. SLC6 neurotransmitter transporters: structure, function, and regulation. Pharmacol. Rev. 63, 585–640.

Kristufek, D., Rudorfer, W., Pifl, C., Huck, S., 2002. Organic cation transporter mRNA and function in the rat superior cervical ganglion. J. Physiol. 543, 117–134.

Liechti, M.E., Baumann, C., Gamma, A., Vollenweider, F.X., 2000. Acute psychological effects of 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") are attenuated by the serotonin uptake inhibitor citalopram. Neuropsychopharmacology 22, 513–521.

Lusthof, K.J., Oosting, R., Maes, A., Verschraagen, M., Dijkhuizen, A., Sprong, A.G., 2011. A case of extreme agitation and death after the use of mephedrone in The Netherlands. Forensic Sci. Int. 206, e93–e95.

Maskell, P.D., De Paoli, G., Seneviratne, C., Pounder, D.J., 2011. Mephedrone (4-methylmethcathinone)-related deaths. J. Anal. Toxicol. 35, 188–191.

Massmann, V., Edemir, B., Schlatter, E., Al-Monajjed, R., Harrach, S., Klassen, P., Holle, S.K., Sindic, A., Dobrivojevic, M., Pavenstadt, H., Ciarimboli, G., 2014. The organic cation transporter 3 (OCT3) as molecular target of psychotropic drugs: transport characteristics and acute regulation of cloned murine OCT3. Pflügers Archiv 466, 517–527.

Mayer, F.P., Wimmer, L., Dillon-Carter, O., Partilla, J.S., Burchardt, N.V., Mihovilovic, M.D., Baumann, M.H., Sitte, H.H., 2016. Phase I metabolites of mephedrone display biological activity as substrates at monoamine transporters. Br. J. Pharmacol. 173, 2657–2668.

Mayer, F.P., Luf, A., Nagy, C., Holy, M., Schmid, R., Freissmuth, M., Sitte, H.H., 2017. Application of a combined approach to identify new psychoactive street drugs and Decipher their mechanisms at monoamine transporters. Curr Top Behav Neurosci 32, 333–350.

Mayer, F.P., Schmid, D., Owens, W.A., Gould, G.G., Apuschkin, M., Kudlacek, O., Salzer, I., Boehm, S., Chiba, P., Williams, P.H., WU, H.H., Gether, U., Koek, W., Daws, L.C., Sitte, H.H., 2018. An unsuspected role for organic cation transporter 3 in the actions of amphetamine. Neuropsychopharmacology PubMed PMID: 29773909.

Meyer, M.R., 2016. New psychoactive substances: an overview on recent publications on their toxicodynamics and toxicokinetics. Arch. Toxicol. 90, 2421–2444.

Murray, B.L., Murphy, C.M., Beuhler, M.C., 2012. Death following recreational use of designer drug "bath salts" containing 3,4-Methylenedioxypyrovalerone (MDPV). J. Med. Toxicol. 8, 69–75.

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.

Ross, E.A., Watson, M., Goldberger, B., 2011. Bath salts" intoxication. N. Engl. J. Med. 365, 967–968.

Salzer, I., Gafar, H., Gindl, V., Mahlknecht, P., Drobny, H., Boehm, S., 2014. Excitation of rat sympathetic neurons via M1 muscarinic receptors independently of Kv7 channels. Pflügers Archiv 466, 2289–2303.

Scholze, P., Zwach, J., Kattinger, A., Pifl, C., Singer, E.A., Sitte, H.H., 2000. Transporter-mediated release: a superfusion study on human embryonic kidney cells stably expressing the human serotonin transporter. J. Pharmacol. Exp. Therapeut. 293, 870–878.

Scholze, P., Sitte, H.H., Singer, E.A., 2001. Substantial loss of substrate by diffusion during uptake in HEK-293 cells expressing neurotransmitter transporters. Neurosci. Lett. 309, 173–176.

Shekar, A., Aguilar, J.I., Galli, G., Cozzi, N.V., Brandt, S.D., Ruoho, A.E., Baumann, M.H., Matthies, H.J.G., Galli, A., 2017. Atypical dopamine efflux caused by 3,4-methylenedioxypyrovalerone (MDPV) via the human dopamine transporter. J. Chem. Neuroanat. 83–84, 69–74.

Simmler, L.D., Buser, T.A., Donzelli, M., Schramm, Y., Dieu, L.H., Huwyler, J., Chaboz, S., Hoener, M.C., Liechti, M.E., 2013. Pharmacological characterization of designer cathinones in vitro. Br. J. Pharmacol. 168, 458–470.

Sitte, H.H., Freissmuth, M., 2015. Amphetamines, new psychoactive drugs and the monoamine transporter cycle. Trends Pharmacol. Sci. 36, 41–50.

Sitte, H.H., Hiptmair, B., Zwach, J., Pifl, C., Singer, E.A., 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.

Spiller, H.A., Ryan, M.L., Weston, R.G., Jansen, J., 2011. Clinical experience with and analytical confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. Clin. Toxicol. 49, 499–505.

Styszko, K., Dudarska, A., Zuba, D., 2016. The presence of stimulant drugs in wastewater from Krakow (Poland): a snapshot. Bull. Environ. Contam. Toxicol. 97, 310–315.

Sulzer, D., Sonders, M.S., Poulsen, N.W., Galli, A., 2005. Mechanisms of neurotransmitter release by amphetamines: a review. Prog. Neurobiol. 75, 406–433.

Tyrkko, E., Andersson, M., kronstrand, R., 2016. The toxicology of new psychoactive substances: synthetic cathinones and phenylethylamines. Ther. Drug Monit. 38, 190–216.

Vialou, V., Balasse, L., Callebert, J., Launay, J.M., Giros, B., Gautron, S., 2008. Altered aminergic neurotransmission in the brain of organic cation transporter 3-deficient mice. J. Neurochem. 106, 1471–1482.

Wu, X., Kekuda, R., Huang, W., Fei, Y.J., Leibach, F.H., Chen, J., Conway, S.J., Ganapathy, V., 1998. Identity of the organic cation transporter OCT3 as the extraneuronal monoamine transporter (uptake2) and evidence for the expression of the transporter in the brain. J. Biol. Chem. 273, 32776–32786.