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The profile of mephedrone on human monoamine transporters differs from 3,4-methylenedioxymethamphetamine primarily by lower potency at the vesicular monoamine transporter

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ABSTRACT

Mephedrone (4-methylmethcathinone, MMC) and 3,4-methylenedioxymethamphetamine (MDMA) are constituents of popular party drugs with psychoactive effects. Structurally they are amphetamine-like substances with monoamine neurotransmitter enhancing actions. We therefore compared their effects on the human monoamine transporters using human cell lines stably expressing the human noradrenaline, dopamine and serotonin transporter (NET, DAT and SERT); preparations of synaptic vesicles from human striatum in uptake experiments; and a superfusion system where releasing effects can be reliably measured. MMC and MDMA were equally potent in inhibiting noradrenaline uptake at NET, with IC_{50} values of 1.9 and 2.1 μM, respectively. Compared to their NET inhibition potency, both drugs were weaker uptake inhibitors at DAT and SERT, with MMC being more potent than MDMA at DAT (IC_{50}: 5.9 vs 12.6 μM) and less potent than MDMA at SERT (IC_{50}: 19.3 vs 7.6 μM). MMC and MDMA both induced concentration-dependently [^{3}H]1-methyl-4-phenylpyridinium-release from NET-, DAT or SERT-expressing cells which was clearly transporter-mediated release as demonstrated by the selective inhibitory effects of nmolar to low μmolar concentrations of desipramine, GBR 12909 and fluoxetine, respectively. MMC and MDMA differed most in their inhibition of [^{3}H]dopamine uptake by synaptic vesicles from human striatum with MDMA being 10-fold more potent than MMC (IC_{50}: 20 vs 223 μM) and their ability to release [^{3}H]dopamine from human vesicular monoamine transporter expressing SH-SY5Y neuroblastoma cells in which MDMA seems to have a stronger effect. Our findings give a molecular explanation to the lower long-term neurotoxicity of MMC compared to MDMA.

1. Introduction

Mephedrone (4-Methylmethcathinone, MMC) and ecstasy (3,4-methylenedioxymethamphetamine, MDMA) are both designer drugs used for illicit recreational consumption due to their psychoactive effects. They are structurally related to amphetamine and thus act as psychostimulants with a risk of addiction. In fact, MMC and MDMA inhibited the uptake of tritiated dopamine, noradrenaline and serotonin into rat brain synaptosomes (Baumann et al., 2013) and induced release from rat brain synaptosomes preloaded with tritiated substrates indicating their status as substrates of the plasmalemmal monoamine transporters (Baumann et al., 2012). These findings suggest a strong monoamine releasing effect of MMC and MDMA on monoaminergic nerve endings which is well supported by studies using in vivo microdialysis (Baumann et al., 2012; Kehr et al., 2011).

Similar to high doses of other releasing drugs repeated MDMA administration induced a selective neurotoxic loss of 5-HT in forebrain regions of the rat and damage to dopamine nerve terminals of the mouse (for review, see Green et al., 2003). By contrast, the majority of studies did not find neurotoxic loss of parameters of serotoninergic or dopaminergic nerve terminals after binge-type dosing schedule of MMC in rats (Baumann et al., 2012; den Hollander et al., 2013; Motbey et al., 2012; Shortall et al., 2012) or mice (Angoa-Perez et al., 2011; den Hollander et al., 2013). Finally, MMC did not activate glia or increase glial fibrillary acidic protein (Angoa-Perez et al., 2011; den Hollander et al., 2013), whereas the latter marker of neurodegeneration was increased by MDMA (Johnson et al., 2002; Miller and O'Callaghan, 1995).

A connection between longterm neurotoxicity of amphetamine-related drugs and interaction with mechanisms regulating the intraneuronal neurotransmitter concentrations has been established. Amphetamine-induced redistribution of dopamine from synaptic vesicles to the cytosol followed by metabolism accompanied by the

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production of radical oxygen species has been hypothesized to trigger nerve terminal loss (Bogen et al., 2003; Cubells et al., 1994; Hansen et al., 2002). The vesicular monoamine transporter 2/SLC18A2 (VMAT2) is a significant regulator of intraneuronal monoamine concentrations and its impairment has recently been implicated in the dopaminergic degeneration in idiopathic Parkinson's disease (Pifl et al., 2014). Since meth/amphetamine users have been shown to have an above-normal risk of developing Parkinson's disease (Callaghan et al., 2012) and even for human MDMA users there are hints for a neurotoxic potential (for review, see Steinkellner et al., 2011), we reasoned that it might be interesting to compare the pharmacology of MMC and MDMA at the human monoamine transporters in uptake and superfusion experiments using transfected cells loaded with the metabolically inert transporter substrate [^{3}H]1-methyl-4-phenylpyridinium (MPP^+) which allows a clear distinction between transport-inhibiting and carrier-mediated outward transport activity of drugs (Scholze et al., 2000) and include experiments on the human vesicular monoamine transporter by taking advantage of our recently reported preparation of functionally active synaptic vesicles from autopsied human striatum (Pifl et al., 2014).

2. Materials and methods

2.1. Materials

Media, sera and other tissue culture reagents were obtained from Life Technologies (Vienna, Austria). [7-^{3}H]dopamine (22 Ci/mmol), levo-[7-^{3}H]noradrenaline (15 Ci/mmol), 5-[1,2-^{3}H[N])-hydroxytryptamine (21 Ci/mmol) were obtained from New England Nuclear GmbH (Vienna, Austria). [^{3}H]1-Methyl-4-phenylpyridinium (MPP^+; 85 Ci/mmol) was supplied by American Radiolabeled Chemicals (St. Louis, MO), desipramine from Ciba-Geigy Limited (Stein, Switzerland), fluoxetine from Eli Lilly and Company Limited (Dublin, Ireland) and mazindol from Sandoz GmbH (Vienna, Austria). The other chemicals were purchased from Sigma-Aldrich or Merck.

2.2. Cell culture

SK-N-MC, SH-SY5Y (human neuroblastoma) and human embryonic kidney (HEK) 293 cells were grown in minimum essential medium with Earle's salts and L-glutamine, 10% heat inactivated fetal bovine serum and 50 mg/l gentamicin. Cells were grown in 100 or 60 mm diameter tissue culture dishes (polystyrene, Falcon) at 37 °C under an atmosphere of 5% CO_2/95% air. The human dopamine transporter/SLC6A3 (DAT) or noradrenaline transporter/SLC6A2 (NET) cDNA was stably expressed in SK-N-MC cells using methods as described previously (Pifl et al., 1996). The human serotonin transporter/SLC6A4 (SERT) was similarly expressed in HEK 293 cells using the vector pRc/CMV and selection by 1 g/l G418 in the medium and the human DAT in SH-SY5Y cells also using the vector pRc/CMV and selection by 0.6 g/l G418.

2.3. Cellular uptake experiments

The cells were seeded in poly-D-lysine-coated 24-well plates (2×10^5 SK-N-MC or 1×10^5 HEK cells/well) and, one day later, each well was washed with 0.5 ml uptake buffer and incubated with 0.5 ml buffer containing various concentrations of the drugs. Uptake was started by addition of [^{3}H]dopamine, [^{3}H]noradrenaline or [^{3}H]serotonin at a final concentration of 1 μM (specific activity 0.14 Ci/mmol) after 2 min of preincubation. After incubation for 2.5 min at 25 °C, it was stopped by aspirating the uptake buffer and washing each well twice with 1 ml icecold buffer. Nonspecific uptake was determined in the presence of 10 μM mazindol (DAT- and NET cells) or 3 μM fluoxetine (SERT-cells). The radioactivity remaining in each well was determined by incubating with 0.4 ml 1% sodium dodecyl sulfate and transferring this solution into scintillation vials containing 3 ml scintillation cocktail (Ultima Gold MV, Packard, Dovners Grove, IL). The uptake buffer consisted of (mmol/l): 4 Tris–HCl; 6.25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 120 NaCl; 5 KCl; 1.2 CaCl2; 1.2 MgSO4; 5.6 D-glucose; 0.5 ascorbic acid; pH 7.1.

2.4. Superfusion experiments

Cells were seeded onto poly-D-lysine-coated 5-mm-diameter glass cover slips in 96-well tissue culture plates (7×10^4 SK-N-MC cells/well, 3×10^4 HEK cells/well and 5×10^4 SH-SY5Y cells/well). On the following morning SK-N-MC and HEK cells were loaded with [^{3}H] MPP^+ in uptake buffer at 37 °C for 20 min: DAT-cells, 6 μM with 0.2 Ci/mmol; NET-cells, 0.1 μM with 29 Ci/mmol; SERT-cells, 10 μM with 0.4 Ci/mmol. SH-SY5Y cells were washed and incubated with serum-free medium one day after seeding and one further day later loaded in uptake buffer with 0.2 μM [^{3}H]dopamine with 40 Ci/mmol at 37 °C for 45 min. After loading, coverslips were transferred to small chambers and superfused (25 °C, 1.0 ml/min) with the uptake buffer mentioned above in a setup as described previously (Pifl et al., 1995; Scholze et al., 2000). After a washout period of 45 min to establish a stable efflux of radioactivity the experiment was started with the collection of 4-min fractions. At the end of the experiment cells were lysed by superfusion with 4 ml 1% SDS. The radioactivity in the superfusate fractions and the SDS-lysates was determined by liquid scintillation counting. Release of tritium was expressed as fractional rate, i.e. the radioactivity released during a fraction was expressed as percentage of the total radioactivity present in the cells at the beginning of that fraction.

2.5. Preparation of synaptic vesicles

Samples of about 600 mg of human striatal tissue from autopsied frozen half brains of control subjects without evidence in their records of any neurological or psychiatric disorder derived from our recent study on vesicular dopamine storage in Parkinson's disease (Pifl et al., 2014) were homogenized in ice-cold 0.3 M sucrose containing 25 mM Tris (pH 7.4) and 10 μM pargyline in a glass Teflon Potter-type homogenizer and vesicles in the supernatants of a P2-pellets of a crude synaptosomal preparation and in H_2O-lysates of P2-pellets were combined as described recently (Pifl et al., 2014) and stored at −80 °C until uptake analysis.

2.6. Vesicular uptake

Uptake was performed in a total volume of 1.5 ml 0.13 M potassium phosphate buffer pH 7.4 containing 2 mM MgATP, 0.1 μM of [^{3}H]dopamine and various concentrations of the uptake interfering drugs. Unspecific uptake was determined in the presence of 1 μM reserpine. Transport was initiated by placing the tubes in a 30 °C water bath and adding 0.5 ml vesicle suspension (obtained from about 20 to 25 mg human tissue) for 4 min. Uptake was terminated by the addition of 2.5 ml ice-cold potassium phosphate buffer, immediate filtration under vacuum onto Whatman GF/B filter paper pre-soaked in 1% polyethylenimine, using a Brandel harvester. The filters were washed twice with additional 3 ml of cold potassium phosphate buffer.

2.7. Data analysis

Uptake data of each separate experiment were fitted to the equation $f=\min+(\max-\min)/(1+x/IC_{50})$, "min" being non-specific uptake, "max" the uptake in the absence of inhibiting drug, x the molar concentration of the inhibiting drug, and IC_{50} the drug concentration that inhibits 50% of specific uptake by the non-linear curve-fitting computer program SigmaPlot (Systat Software, Inc., CA, U.S.A). min was constrained to nonspecific uptake. All results were expressed as means ± S.E.M.

3. Results

3.1. Inhibition of NET-, DAT- and SERT-mediated uptake by MMC and MDMA

In cells stably expressing the human NET, DAT or SERT, MMC and MDMA concentration-dependently inhibited transport of tritiated noradrenaline, dopamine or serotonin, respectively (Fig. 1). Whereas both MMC and MDMA displayed the highest and about equal potency at the NET (IC_{50}, MMC: 1.9 ± 0.7 μM, $n=5$; MDMA: 2.1 ± 0.3 μM, $n=5$), the rank order of potency was different at the DAT and SERT with MMC being clearly more potent than MDMA at the DAT (IC_{50}, MMC: 5.9 ± 1.4 μM, MDMA: 12.6 ± 1.6 μM, $n=4$) the opposite rank order of potency at the SERT (IC_{50}, MMC: 19.3 ± 1.0 μM, $n=5$; MDMA: 7.6 ± 1.2 μM, $n=5$).

3.2. Stimulation of NET, DAT and SERT-mediated release by MMC and MDMA

In cells expressing the human NET, DAT and SERT preloaded with the metabolically inert transporter substrate [^{3}H]MPP^+, grown on cover-slips and superfused in microchambers, MMC and MDMA added to the superfusion buffer concentration-dependently increased radioactivity in the fractionated perfusates in a completely reversible manner (Figs. 2 and 3, respectively). At all transporters, a releasing effect of MMC was clearly discernable at 0.1 μM and the maximum effect leveled off at concentrations between 10 and 30 μM. A releasing effect of MDMA was obvious at 0.1 μM at the NET and SERT, but the effect of 0.1 μM MDMA was not different from vehicle at the DAT; again there was no further increasing effect between 10 and 30 μM at all transporters with even a slightly lower maximum of MDMA at 30 μM than at 10 μM in SERT expressing cells.

3.3. Blocking action of selective inhibitors of monoamine transporters on MMC-induced release

The highly potent NET blocker desipramine, added at the concentration of 0.03 or 0.3 μM to the superfusion buffer at min 8, concentration-dependently blocked the releasing action of 10 μM MMC in NET-cells with a clear inhibitory action already to be seen at 0.03 μM (Fig. 4A). There was no effect of 0.03 or 0.3 μM desipramine on the releasing effect of 10 μM MMC in DAT cells (Fig. 4B) whereas in SERT cells 0.03 μM desipramine was without effect and 0.3 μM desipramine partially prevented the effect of 10 μM MMC (Fig. 4C).

The experimental DAT blocker GBR12909, added at the concentration of 0.01 or 0.1 μM to the superfusion buffer at min 8, was without effect on the efflux stimulatory effect of 10 μM MMC in NET (Fig. 5A) and SERT cells (Fig. 5C), but GBR12909 blocked the MMC effect in DAT cells partially at the concentration of 0.01 μM and completely at 0.1 μM (Fig. 5C).

The first selectively serotonin transport inhibiting and widely used antidepressant fluoxetine, added at the concentration of 0.1 or 1 μM, was without effect on the efflux stimulatory effect of 10 μM MMC in NET cells (Fig. 6A), had a minor but not concentration-dependent inhibitory effect in DAT cells (Fig. 6B), but completely blocked the releasing effect of MMC at both concentrations on SERT cells (Fig. 6C).

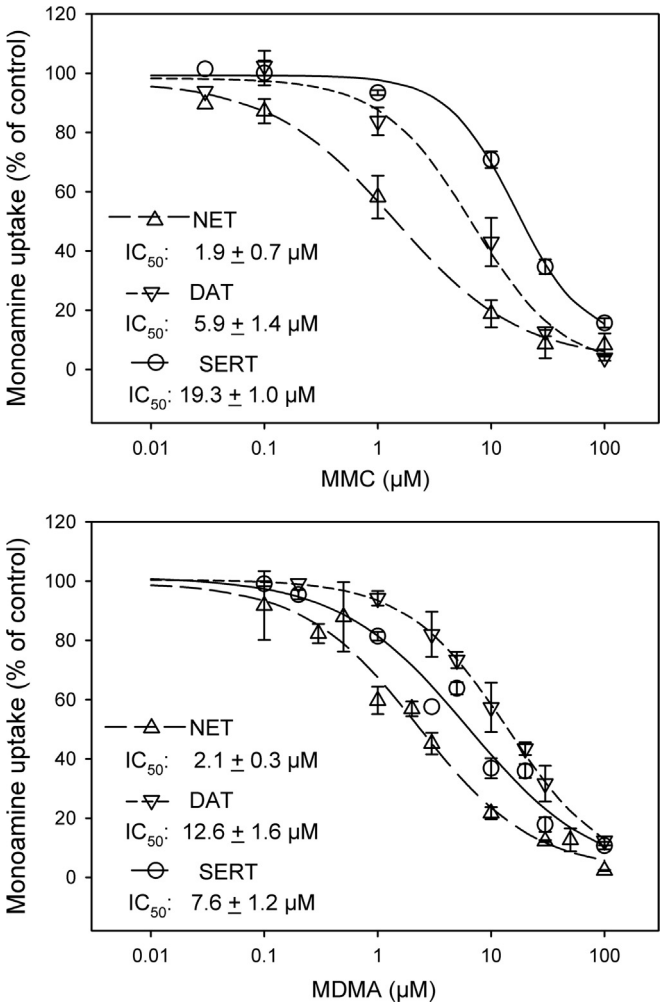

Fig. 1. Effects of MMC and MDMA on uptake by the human NET, DAT and SERT. Concentration-inhibition curves on [^{3}H]noradrenaline, [^{3}H]dopamine and [^{3}H]serotonin uptake in SK-N-MC or HEK293 cells stably expressing the NET (triangles up, long dash), DAT (triangles down, short dash) or SERT (circles, solid). The cells were incubated in 24-well plates for 2.5 min at 25 °C with 1 μM of the tritiated monoamines in the absence (control) or presence of MMC (A) or MDMA (B) at the concentrations indicated, and uptake was determined as described in Materials and Methods. Symbols represent means ± S.E.M. of three to five independent experiments, each in duplicates. The data of each experiment were fitted by nonlinear regression and the mean of the IC_{50} values ± S.E.M. are inserted into the panels.

3.4. Inhibition of dopamine uptake into synaptic vesicles from human striatum by MMC and MDMA

Uptake of [^{3}H]dopamine into synaptic vesicles prepared from human caudate or putamen shows all features of uptake driven by the VMAT2 in terms of ATP-dependence and activity in completely sodium-free potassium phosphate buffer which rules out interfering effects on sodium- and chloride-dependent DAT, SERT or NET (Pifl et al., 2014). Reserpine concentration-dependently blocked uptake with its well established high potency in the nanomolar range (IC_{50}: 5.5 ± 1.5 nM, $n=3$; Fig. 7). MMC and MDMA inhibited dopamine uptake with a much lower potency which differed between MMC and MDMA by a factor of 10 (IC_{50}, MMC: 223 ± 55 μM, $n=5$; MDMA: 20 ± 6 μM, $n=5$; Fig. 7).

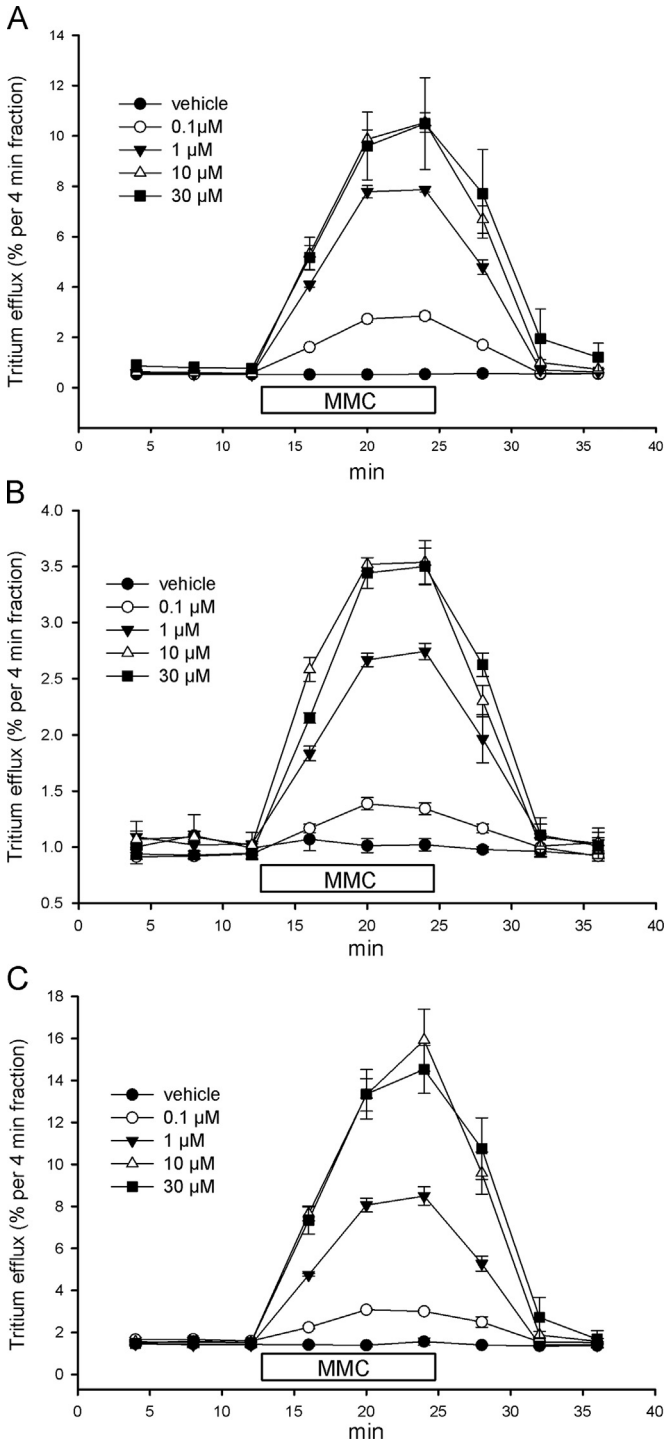

Fig. 2. Concentration-dependent effect of MMC on release by the human NET, DAT and SERT. SK-N-MC or HEK293 cells stably expressing the NET (A), DAT (B) or SERT (C) were loaded with [^{3}H]MPP$^+$, superfused and 4-min fractions were collected. After three fractions (12 min) of basal efflux, cells were exposed for three fractions (bar) to buffers containing vehicle (filled circle) or different concentrations of MMC (0.1 μM, open circle; 1 μM, filled triangle down; 10 μM, open triangle up; 30 μM, filled square). Data are presented as fractional efflux, i.e., each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± S.E.M. of three to four independent experiments.

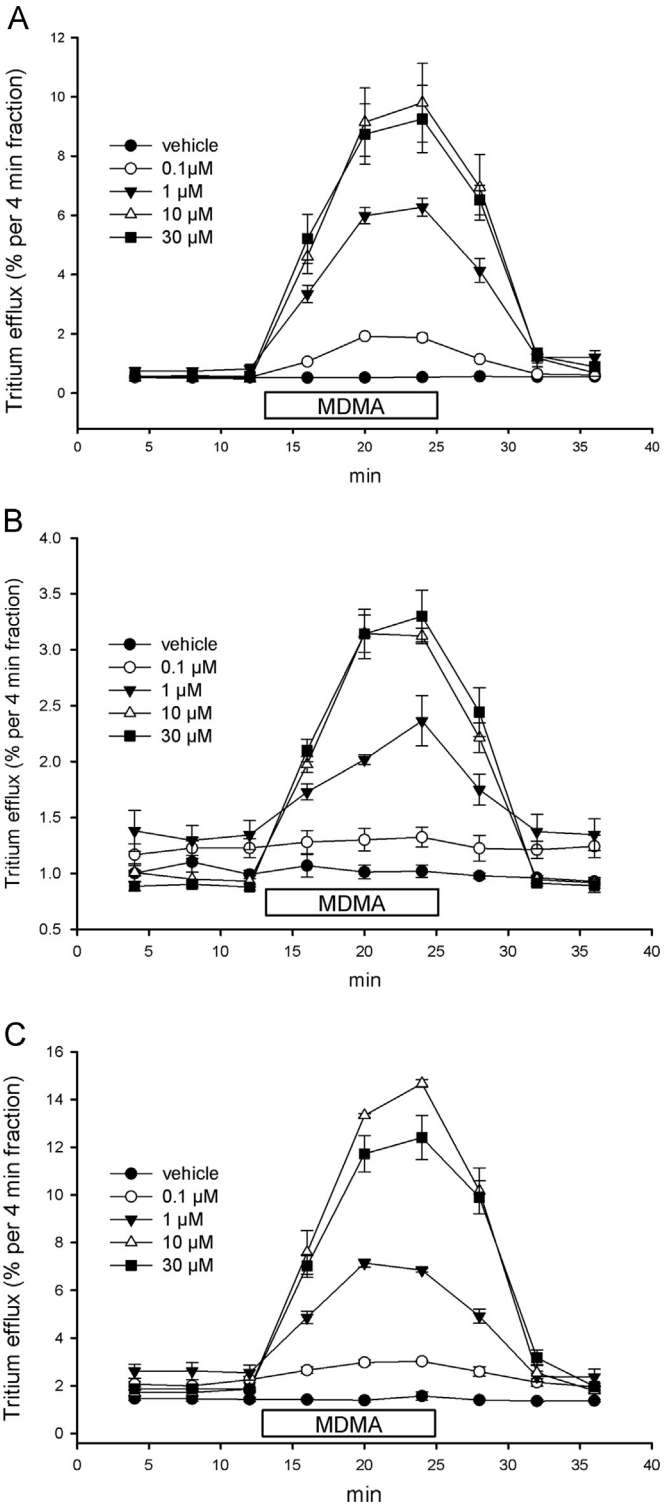

Fig. 3. Concentration-dependent effect of MDMA on release by the human NET, DAT and SERT. SK-N-MC or HEK293 cells stably expressing the NET (A), DAT (B) or SERT (C) were loaded with [^{3}H]MPP$^+$, superfused and 4-min fractions were collected. After three fractions (12 min) of basal efflux, cells were exposed for three fractions (bar) to buffers containing vehicle (filled circle) or different concentrations of MDMA (0.1 μM, open circle; 1 μM, filled triangle down; 10 μM, open triangle up; 30 μM, 30 μM, filled square). Data are presented as fractional efflux, i.e., each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± S.E.M. of three to four independent experiments.

3.5. *Stimulation of DAT-mediated release in [^{3}H]dopamine loaded SH-SY5Y neuroblastoma cells by MMC and MDMA*

The human neuroblastoma SH-SY5Y express many properties of monoaminergic neurons including potassium and veratridine-evoked monoamine release and the VMAT2 (Murphy et al., 1991; Watabe and Nakaki, 2008). We stably transfected them with the human DAT, grew them on cover-slips, loaded them with

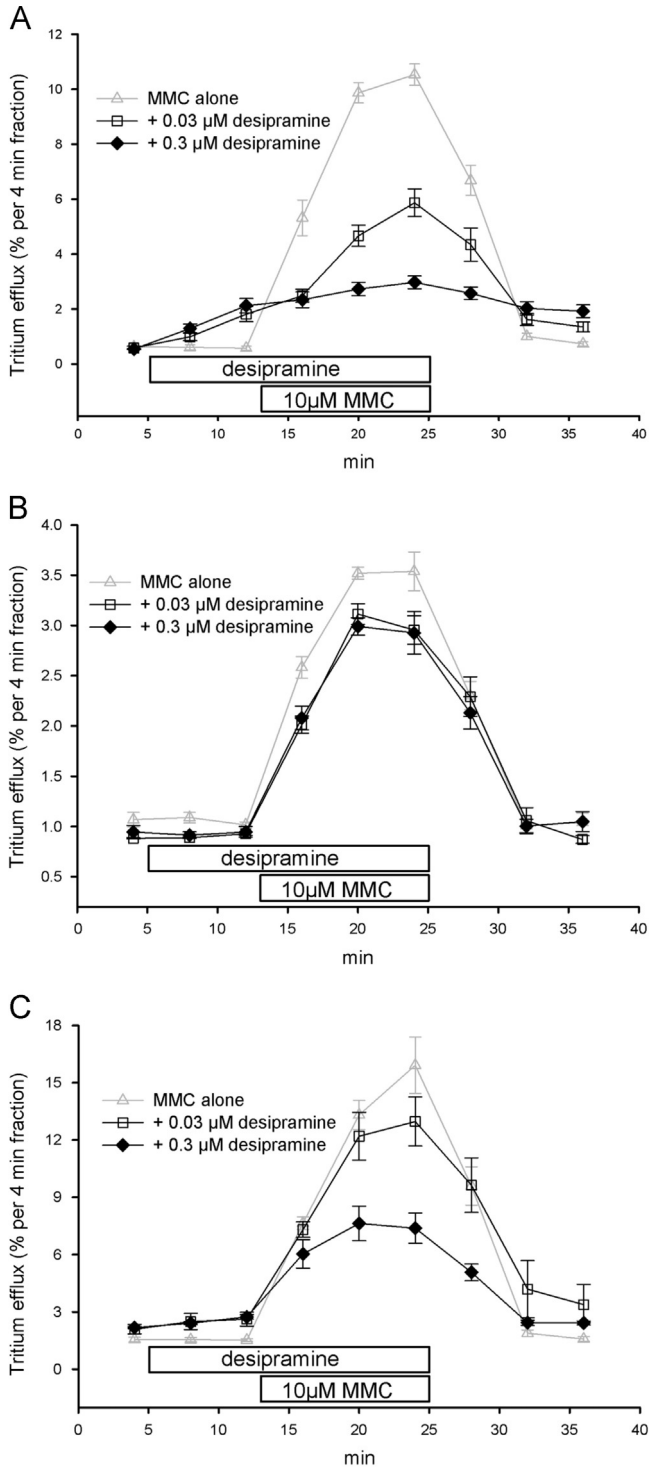

Fig. 4. Effects of desipramine on MMC-induced release by the human NET, DAT and SERT. SK-N-MC or HEK293 cells stably expressing the NET (A), DAT (B) or SERT (C) were loaded with [^{3}H]MPP$^+$, superfused and 4-min fractions were collected. After one fraction of basal efflux, cells were exposed to buffers containing either 0.03 μM (4 min, upper bar, open squares) or 0.3 μM desipramine (4 min, upper bar, filled diamonds). Two fractions later (12 min, lower bar), the superfusion was switched for three fractions to a buffer containing additional 10 μM MMC. Data on the release by 10 μM MMC (gray triangles up) are included for comparison from Fig. 2. Data are presented as fractional efflux, i.e., each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± S.E.M. of three to four independent experiments.

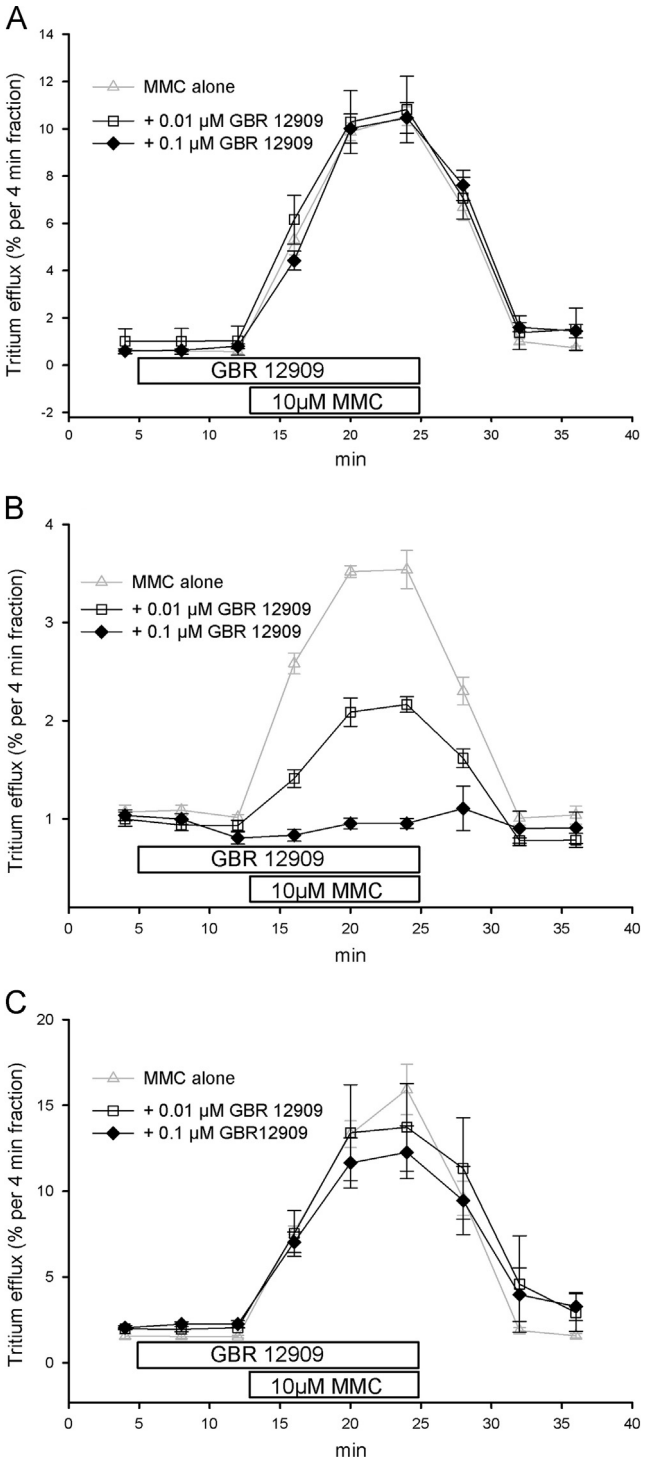

Fig. 5. Effects of GBR 12909 on MMC-induced release by the human NET, DAT and SERT. SK-N-MC or HEK293 cells stably expressing the NET (A), DAT (B) or SERT (C) were loaded with [^{3}H]MPP$^+$, superfused and 4-min fractions were collected. After one fraction of basal efflux, cells were exposed to buffers containing either 0.01 μM (4 min, upper bar, open squares) or 0.1 μM GBR 12909 (4 min, upper bar, filled diamonds). Two fractions later (12 min, lower bar), the superfusion was switched for three fractions to a buffer containing additional 10 μM MMC. Data on the release by 10 μM MMC (gray triangles up) are included for comparison from Fig. 2. Data are presented as fractional efflux, i.e., each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± S.E.M. of three to four independent experiments.

[^{3}H]dopamine and superfused them in microchambers. MDMA and MMC were added at a concentration of 10 or 30 μM to the superfusion buffer; MDMA (Fig. 8B) concentration-dependently increased radioactivity in the fractionated perfusates whereas MMC (Fig. 8A) was without effect at 10 μM and stimulated efflux with significantly weaker activity than MDMA at 30 μM (increase of tritium efflux at

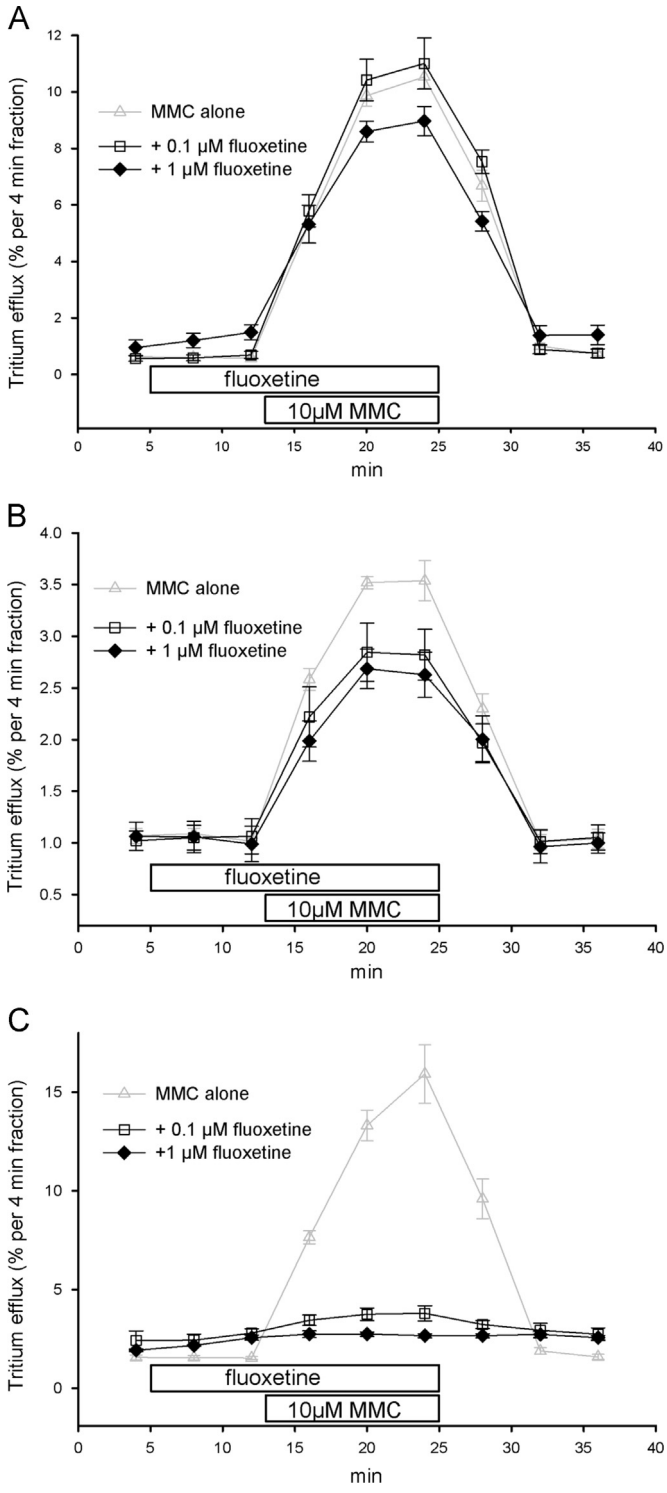

Fig. 6. Effects of fluoxetine on MMC-induced release by the NET, DAT and SERT. SK-N-MC or HEK293 cells stably expressing the NET (A), DAT (B) or SERT (C) were loaded with [^{3}H]MPP$^+$, superfused and 4-min fractions were collected. After one fraction of basal efflux, cells were exposed to buffers containing either 0.1 μM (4 min, upper bar, open squares) or 1 μM fluoxetine (4 min, upper bar, filled diamonds). Two fractions later (12 min, lower bar), the superfusion was switched for three fractions to a buffer containing additional 10 μM MMC. Data on the release by 10 μM MMC (gray triangles up) are included for comparison from Fig. 2. Data are presented as fractional efflux, i.e., each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± S.E.M. of three to four independent experiments.

20 min over the mean baseline values of the first three fractions: 30 μM MDMA, 0.210 ± 0.029%; 30 μM MMC, 0.092 ± 0.010%; $n=5$, $P<0.001$).

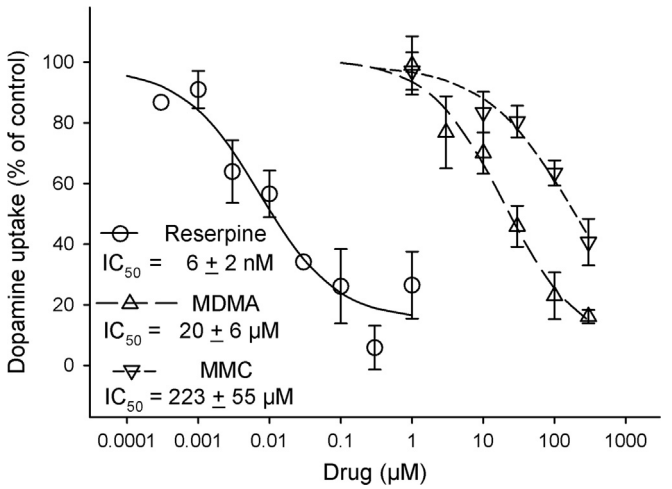

Fig. 7. Effects of reserpine, MDMA and MMC on the uptake by the human VMAT2. Concentration-inhibition curves on dopamine uptake in synaptic vesicles from the human striatum. The vesicle preparations were incubated in potassium phosphate buffer for 4 min at 30 °C with 0.1 μM [^{3}H]dopamine in the absence (control) or presence of reserpine (circles, solid line), MDMA (triangles up, long dash) or MMC (triangles down, short dash) at the concentrations indicated, and uptake was determined as described in Section 2. Symbols represent means ± S.E.M. of three to five independent experiments, each in duplicates. The data of each experiment were fitted by nonlinear regression and the mean of the IC_{50} values ± S.E.M. are inserted into the graph.

4. Discussion

The main finding of our study is that with regard to their effects on the human brain monoamine transporters, MMC and MDMA differ from each other primarily in their interaction with the human brain vesicular monoamine uptake, with MMC having a tenfold lower transport inhibitory potency than MDMA. The differences in inhibition of uptake by plasmalemmal transporters are much smaller, with twofold higher potency of MMC at the DAT and about threefold higher potency of MDMA at the SERT, a rank order in agreement with that in recent studies on human transporters in HEK cells (Eshleman et al., 2013; Iversen et al., 2013; Simmler et al., 2013). The fact that the lower potency of MMC at the VMAT2 went hand in hand with a significantly lower dopamine releasing action of MMC vs. MDMA in a human neuroblastoma cell line shows that dopamine release from [^{3}H]dopamine loaded cells requires dual action by the drug, i.e. dopamine mobilization from intracellular stores to the cytosol by VMAT2 inhibition and reverse transport induction by plasmalemmal DAT interaction. The differences on DA release in DAT expressing SH-SY5Y demonstrate differences in interaction with VMAT2 in a cellular setting beyond that on isolated synaptic vesicles and are by no means relevant for differences in releasing abilities on dopaminergic neurons in vivo which are clearly dominated by the high potency of MMC at the DAT (Baumann et al., 2012; Kehr et al., 2011; this study), which is again consistent with the higher abuse liability of MMC (for review, see Green et al. (2014)). The weaker interaction with the vesicular monoamine transporter of MMC however might be a potential explanation for the lack of long term depletion of brain monoamines and the lack of other signs of neurotoxicity in binge-type repeated administration of MMC in rodent experiments. Our findings on human tissue and cultured cells appear significant considering that chronic toxicity of amphetamine-related drugs cannot be excluded even in human users (for review, see Steinkellner et al. (2011)) and because increased risk of Parkinson's disease has been related to the use of methamphetamine or other amphetamine-type drugs (Callaghan et al., 2012).

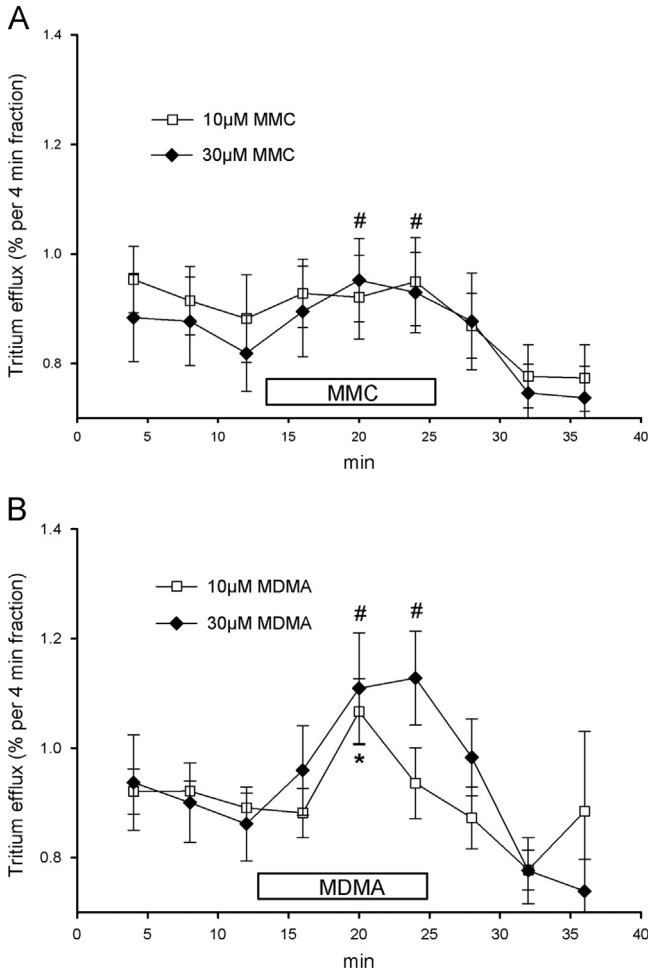

Fig. 8. Effect of MMC and MDMA on the dopamine efflux of dopaminergic cells. Human SH-SY5Y neuroblastoma cells stably expressing the human DAT were loaded with [^{3}H]dopamine, superfused and 4-min fractions were collected. After three fractions (12 min) of basal efflux, cells were exposed for three fractions (bar) to buffers containing MMC (A) or MDMA (B) at different concentrations (10 μM, open squares; 30 μM, filled diamonds). Data are presented as fractional efflux, i.e., each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± S.E.M. of three to four independent experiments. $^*P < 0.05$ 10 μM MMC, $^\#P < 0.005$ 30 μM MMC or MDMA vs mean value of fractions 1–3 by paired Student's *t*-test.

The mechanism of long-term neurotoxicity of amphetamine-related drugs is still unclear. It was proposed that mobilization of monoamines from synaptic vesicles by altering vesicular pH or by direct VMAT2 interaction together with concurrent deficits of plasmalemmal transporter function after repeated administration of amphetamines might trap the neurotransmitter in the cytosol because of increased efflux from vesicles into the cytosol parallel to reduced efflux from the cytosol to the extracellular space (Hansen et al., 2002). Especially excessive cytosolic dopamine might damage nerve terminals by increased formation of dopamine-associated oxygen radicals and reactive metabolites, and serotonin oxidation by monoamine oxidase was also proposed to cause free oxygen radical formation (Bogen et al., 2003). Such a mechanism is unlikely for MMC which inhibited uptake into synaptic vesicles only at high micromolar concentrations and only weakly mobilized [^{3}H]dopamine from neuroblastoma cells. As a matter of fact, sustained release of preloaded [^{3}H]dopamine from superfused DAT expressing cells by amphetamine was shown to be dependent on mobilization of a vesicular pool after additional VMAT2 transfection (Pifl et al., 1995). A more than tenfold higher potency of MDMA than the MMC related methcathinone and methylone was also reported recently in bovine chromaffine granules (Cozzi et al., 1999) and than MMC itself for inhibition of reserpine sensitive uptake of [^{3}H]serotonin into membranous structures released by osmotic shock from HEK293 cells stably transfected with the human VMAT2 cDNA (Eshleman et al., 2013). The EC_{50} value for MMC-induced release of [^{3}H]noradrenaline at this VAMT2 preparation was in fact insignificantly lower than that of MDMA, however the maximum release was only half of it (Eshleman et al., 2013). A rather high potency of MMC was reported for inhibition of [^{3}H]dopamine uptake into synaptic vesicles prepared from rat striatal synaptosomes, but no comparative data were shown for MDMA in this study (López-Arnau et al., 2012). Apart from this potential species difference, the rank order of potency of MMC and MDMA was rather similar in DAT, NET and SERT assays obtained from rat tissue and in cells transfected with the respective human transporter cDNA with similar potency of MMC and MDMA at the NET, higher potency of MMC than MDMA at the DAT and the opposite rank order at the SERT (Baumann et al., 2012, 2013; Eshleman et al., 2013; Hadlock et al., 2011; Iversen et al., 2013; Simmler et al., 2013).

Our interaction experiments with specific uptake blockers clearly demonstrate that MMC-induced release of [^{3}H]MPP^+ from NET-, DAT- or SERT-expressing cells was solely due to carrier-mediated reverse transport via the plasmalemmal transporters: in fact, specific concentrations of GBR12909 and fluoxetine were found that completely suppressed MMC-induced release from DAT- and SERT-cells, respectively, leaving release via the other transporters unaffected. [^{3}H]MPP^+ is also a substrate of non-neuronal monoamine transporters (Grundemann et al., 1999) which are found endogenously in kidney and neuroblastoma tissue (Bayer et al., 2009; Fujita et al., 2006) and our interaction experiments could rule out their potential contributions to releasing effects of MMC and MDMA studied in kidney and neuroblastoma derived HEK293 or SK-N-MC cells.

The ability of a drug to induce transporter-mediated release can be detected most reliably in superfusion experiments on cells expressing the relevant transporters. Even in these experiments interference of a pseudo-releasing action must be taken into account. Such a pseudo-releasing action is due to inhibition by the drug of re-uptake of substrate released upstream by diffusion (Scholze et al., 2000) or by another transporter such as an organic cation transporter (Kristufek et al., 2002). In our study this was minimized by superfusion of cells sparsely seeded on coverslips and loaded with a transporter substrate that is metabolically inert, hydrophilic due to a permanent charge and has a relatively low affinity to the transporter on which reuptake blockade might mimic transporter-mediated release. The metabolically inert and permanently charged MPP^+ is an ideal transporter substrate on the DAT and the SERT with K_M values in uptake experiments above 7 μM, but not on the NET where the K_M is around 0.8 μM (Pifl et al., 1996). A pseudo-releasing action by inhibiting re-uptake of released [^{3}H]MPP^+ at the NET may therefore be seen in Fig. 4A as a small increase of tritium induced by the pure uptake inhibitor desipramine in the fractions at min 8 and 12 and an efflux of 10 μM MMC even in the presence of 0.3 μM desipramine above baseline. By contrast, because MPP^+ has only low affinity to DAT and SERT transporters, the DAT inhibitor GBR 12909 and the SERT inhibitor fluoxetine did not affect basal efflux of [^{3}H]MPP^+, while blocking the MMC-induced efflux to virtually baseline in DAT- and SERT-cells, respectively (Fig. 5B and C). MMC induced release of [^{3}H]MPP^+ from DAT expressing SK-N-MC cells equally well as MDMA, but was less effective than MDMA in inducing release of [^{3}H]dopamine from DAT expressing SH-SY5Y cells. The reason lies in the high cytosolic concentration of the metabolically inert [^{3}H]MPP^+ which can be immediately released from the cell by MMC or MDMA via the plasmalemmal transporter acting in reverse. By

contrast, [^{3}H]dopamine has low cytosolic concentrations due to cytosolic degradation and has to be mobilized from storage site of the SH-SY5Y cells to the cytosol by action on the VMAT2 before it can be released by the DAT; as mentioned, MMC acts on the VMAT2 much less effectively than MDMA. The greater extracellular dopamine increase by MMC than MDMA reported in microdialysed nucleus accumbens at equal dosage (Kehr et al., 2011) might be due to findings that amphetamine effects on release in vivo are predominantly driven by DAT blockade (Daberkow et al., 2013).

In conclusion, MMC and MDMA are both able to reverse translocation by human plasmalemmal monoamine transporters. They act most potently at the NET as it was also reported for other amphetamine-type stimulants in preparations from the rat (Rothman et al., 2001), and it can be expected that release of noradrenaline contributes to the cardiovascular and stimulant-like effects of MMC in humans as already shown for MDMA (Hysek et al., 2011, 2013) and psychostimulants in general (Schmidt and Weinshenker, 2014). MMC and MDMA differ from each other particularly in their ability to mobilize intracellular monoamines from synaptic vesicles, with a tenfold lower potency of MMC in inhibiting the human VMAT2. This low potency of MMC on the VMAT2 might make its long-term neurotoxic action, still not to be ruled out for MDMA, less likely.

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