

# The psychostimulant ( $\pm$ )-*cis*-4,4'-dimethylaminorex (4,4'-DMAR) interacts with human plasmalemmal and vesicular monoamine transporters

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## ABSTRACT

( $\pm$ )-*cis*-4,4'-Dimethylaminorex (4,4'-DMAR) is a new psychoactive substance (NPS) that has been associated with 31 fatalities and other adverse events in Europe between June 2013 and February 2014. We used *in vitro* uptake inhibition and transporter release assays to determine the effects of 4,4'-DMAR on human high-affinity transporters for dopamine (DAT), norepinephrine (NET) and serotonin (SERT). In addition, we assessed its binding affinities to monoamine receptors and transporters. Furthermore, we investigated the interaction of 4,4'-DMAR with the vesicular monoamine transporter 2 (VMAT2) in rat pheochromocytoma (PC12) cells and synaptic vesicles prepared from human striatum. 4,4'-DMAR inhibited uptake mediated by human DAT, NET or SERT, respectively in the low micromolar range (IC<sub>50</sub> values < 2  $\mu$ M). Release assays identified 4,4'-DMAR as a substrate type releaser, capable of inducing transporter-mediated reverse transport via DAT, NET and SERT. Furthermore, 4,4'-DMAR inhibited both the rat and human isoforms of VMAT2 at a potency similar to 3,4-methylenedioxymethylamphetamine (MDMA). This study identified 4,4'-DMAR as a potent non-selective monoamine releasing agent. In contrast to the known effects of aminorex and 4-methylaminorex, 4,4'-DMAR exerts profound effects on human SERT. The latter finding is consistent with the idea that fatalities associated with its abuse may be linked to monoaminergic toxicity including serotonin syndrome. The activity at VMAT2 suggests that chronic abuse of 4,4'-DMAR may result in long-term neurotoxicity.

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**Abbreviations:** 4,4'-DMAR, ( $\pm$ )-*cis*-4,4'-dimethylaminorex; 4-MAR, 4-methylaminorex; 5-HT, serotonin; DAT, dopamine transporter; GAT1, *gamma*-aminobutyric acid (GABA) transporter subtype 1; MDMA, 3,4-methylenedioxymethylamphetamine; MPP<sup>+</sup>, 1-methyl-4-phenylpyridinium; NET, norepinephrine transporter; SERT, 5-HT transporter; TAAR1, Trace amine-associated receptor 1; VMAT2, vesicular monoamine transporter 2.

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## 1. Introduction

In recent years, an increasing number of new psychoactive substances (NPS) appeared on the recreational drug markets. NPS mimic the effects of established drugs of abuse, ranging from cannabinoids to psychostimulants (UNODC, 2017). Typically, the pharmacology is poorly characterized and adverse clinical effects are often associated with their abuse (Baumann and Volkow, 2016). ( $\pm$ )-*cis*-4,4'-dimethylaminorex (( $\pm$ )-*cis*-4-methyl-5-(4-methylphenyl)-4,5-dihydro-1,3-oxazol-2-amine, 4,4'-DMAR) represents a

prime example of an abused substance for which scientific literature is scarce. 4,4'-DMAR is derived from drug development efforts in the search for potential anorectic medicines such as aminorex (Poos et al., 1963). In 2012, 4,4'-DMAR was identified in the Netherlands and reported to the European Monitoring Center for Drugs and Drug Addiction (EMCDDA). Between June 2013 and February 2014, 4,4'-DMAR has been analytically confirmed and linked to 31 deaths (EMCDDA, 2015; Brandt et al., 2014).

So far, the chemical features of 4,4'-DMAR and its releasing activity at monoamine transporters in rat brain synaptosomes have been reported (Brandt et al., 2014; McLaughlin et al., 2015). Psychostimulants that target the high-affinity transporters for monoamines can act in two different modes: as (i) non-transported inhibitors (e.g. cocaine) or (ii) they can be recognized as substrates, often referred to as “amphetamine-type” drugs or “releasers” (Sitte and Freissmuth, 2015). The latter “invert” the physiological transport direction of the transporters which results in transporter-mediated release of neurotransmitters. It has been reported that 4,4'-DMAR induced release via rat DAT (SLC6A3), NET (SLC6A2) and SERT (SLC6A4) and hence, the compound was classified a “releaser” (Brandt et al., 2014).

In comparison to its congeners aminorex and 4-MAR ((±)-*cis*-4-methylaminorex), 4,4'-DMAR has been shown to be a more potent releaser at SERT (Brandt et al., 2014). The pharmacological effects of all three compounds resemble those of amphetamine (Hofmaier et al., 2014; Kankaanpää et al., 2001; Rothman et al., 1999). However, aminorex (DAT/SERT ratio = 45) and 4-MAR (DAT/SERT ratio = 31) were found to be more catecholamine-selective compared to 4,4'-DMAR (DAT/SERT ratio = 2) (Brandt et al., 2014).

In 2014, a risk assessment carried out on 4,4'-DMAR by the EMCDDA revealed that the clinical features associated with the reported adverse effects were consistent with serotonin and cardiovascular toxicity and that combinations of 4,4'-DMAR with various other drugs also targeting the monoaminergic system were thought to have played a contributory role in the fatalities (EMCDDA, 2015). During the following years, 4,4'-DMAR was critically reviewed by the World Health Organization and subsequently controlled internationally and listed in Schedule II of the Convention on Psychotropic Substances of 1971 (UNODC, 2016).

In the present study, we attempted to provide the first complete pharmacological characterization of 4,4'-DMAR's interaction with human plasmalemmal monoamine transporters: Specifically, we examined the interplay between 4,4'-DMAR and the most relevant monoamine receptors and transporters in terms of uptake inhibition and releasing action, as well as their binding profile. Furthermore, we performed two different approaches to investigate the inhibitory properties of 4,4'-DMAR at human and rat VMAT2. Perturbations of VMAT2 function have been associated with neurotoxicity and depletion of brain monoamines (German et al., 2015). Considering the inhibitory effects at VMAT2 and its activity as substrate at DAT, NET and SERT may provide a molecular explanation for the adverse clinical effects associated with 4,4'-DMAR.

## 2. Materials and methods

### 2.1. Reagents and chemicals

(±)-*cis*-4,4'-DMAR hydrochloride was available from previous studies (Brandt et al., 2014). Reagents used in the experiments for uptake inhibition and release in HEK293 cells were used as described in Hofmaier et al. (2014). For uptake and release experiments [<sup>3</sup>H]1-methyl-4-phenylpyridinium ([<sup>3</sup>H]MPP<sup>+</sup>; 80–85 μCi x mmol<sup>-1</sup>) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [<sup>3</sup>H]Dopamine (55 μCi x mmol<sup>-1</sup>), [<sup>3</sup>H]GABA (89,7 μCi x mmol<sup>-1</sup>), [<sup>3</sup>H]5-HT (28.3 μCi x mmol<sup>-1</sup>), [<sup>3</sup>H]8-OH-

DPAT, [<sup>3</sup>H]ketanserin, [<sup>3</sup>H]mesulergine, [<sup>3</sup>H]prazosin, [<sup>3</sup>H]rauwolscine, [<sup>3</sup>H]spiperone, N-methyl-[<sup>3</sup>H]nisoxetine, [<sup>3</sup>H]WIN35,428, and [<sup>3</sup>H]citalopram were all from Perkin Elmer (Boston, MA, USA). [<sup>3</sup>H]RO5166017 and RO5166017 were provided from F. Hoffmann-La Roche (Basel, Switzerland). All other chemicals and cell culture supplies were ordered from Sigma-Aldrich (St. Louis, MO, USA) with the exception of cell culture dishes, which were obtained from Sarstedt (Nuembrecht, Germany).

### 2.2. Cell culture

Human embryonic kidney (HEK293) cells, used for uptake inhibition and superfusion release assays, were maintained in a humidified atmosphere (37 °C and 5% CO<sub>2</sub>) at a subconfluent state in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), penicillin (100 U x 100 mL<sup>-1</sup>) and streptomycin (100 μg x 100 mL<sup>-1</sup>). Geneticin (50 μg x mL<sup>-1</sup>) was added to maintain the selection process. The generation of stable, monoclonal cell lines is described elsewhere (Mayer et al., 2016a). Twenty-four hours prior to uptake inhibition experiments, HEK293 cells expressing the desired transporter, were seeded at a density of 40,000 cells per well onto poly-D-lysine (PDL) coated 96-well plates in a final volume of 200 μL per well. Analogously, 24 h before release experiments, 40,000 cells per well were seeded onto poly-D-lysine coated glass coverslips (5 mm in diameter), that have been placed into a 96-well plate for a final volume of 200 μL per well (Mayer et al., 2016a).

Cells used for receptor and transporter binding assays and receptor activation assays, were cultured and prepared as recently described in detail (Luethi et al., 2017a). For membrane preparations, the cells were harvested following application of trypsin/ethylenediaminetetraacetic acid (EDTA), washed with ice-cold PBS, pelleted via centrifugation (1000 rpm for 5 min at 4 °C), frozen and stored at -80 °C. The frozen pellets were then suspended in 20 mL (receptor binding) or 400 mL (transporter binding) HEPES-NaOH (20 mM, pH 7.4) containing 10 mM EDTA. After homogenization at 14,000 rpm for 20 s (receptor binding) or 10,000 rpm for 15 s (transporter binding), the homogenates were centrifuged at 48,000 g and 4 °C for 30 min. For receptor binding assays, supernatants were discarded and the pellets resuspended in 20 mL HEPES-NaOH (20 mM, pH 7.4) containing 0.1 mM EDTA and homogenized at 14,000 rpm for 20 s. The centrifugation and removal of the supernatant was repeated, and the final pellet was resuspended in HEPES-NaOH that contained 0.1 mM EDTA and homogenized. The following transfected cell lines were used for the binding assays: HEK293 cells (5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, TAAR1, D<sub>2</sub>, hDAT, hNET, and hSERT), Chinese hamster ovary (CHO) cells (α<sub>1A</sub>), and Chinese hamster lung (CHL) cells (α<sub>2A</sub>).

Rat pheochromocytoma cells (rPC12), used for VMAT2 uptake inhibition assays, were grown in PDL-coated cell culture dishes (10 cm diameter) in Opti-MEM (Gibco), supplemented with 5% Fetal Bovine Serum (FBS) and 10% horse serum, penicillin (100 U x 100 mL<sup>-1</sup>) and streptomycin (100 μg x 100 mL<sup>-1</sup>). For VMAT2-assays, the cells were seeded at 40,000 cells per well onto PDL coated 96-well plates in a final volume of 200 μL per well 24 h beforehand.

The human striatal tissue was derived from autopsied frozen half brains of subjects without evidence of any neurological or psychiatric disorder in their records as described earlier (Piffl et al., 2015).

### 2.3. Uptake inhibition assays

For uptake inhibition experiments, DMEM was removed from the cell culture dishes and replaced with Krebs-HEPES-buffer (KHB;

25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM CaCl<sub>2</sub>, and 1.2 mM MgSO<sub>4</sub> and 5 mM *D*-glucose, pH adjusted to 7.3) at a final volume of 200  $\mu$ L per well. The cells were exposed to increasing concentrations of 4,4'-DMAR, diluted in KHB, and 3,4-methylenedioxymethylamphetamine (MDMA), diluted in Milli-Q H<sub>2</sub>O, at a final volume of 200  $\mu$ L per well for 10 min to ensure equilibrated conditions. This was the case for hSERT, hDAT, hNET transfected HEK cells as well as rat GAT1 (SLC6A1), expressed in HEK293 cells. For the 4,4'-DMAR experiments, tritiated substrate (0.2  $\mu$ M [<sup>3</sup>H]5-HT for hSERT, 0.01  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup> for hDAT and hNET, 0.15  $\mu$ M [<sup>3</sup>H]GABA for rGAT1) was added after 10 min. Tritiated substrate utilized in the MDMA uptake inhibition assays was 0.2  $\mu$ M [<sup>3</sup>H]5-HT for hSERT, 0.2  $\mu$ M [<sup>3</sup>H]DOP for hDAT and 0.015  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup> for hNET. Uptake was terminated after 60 s for hSERT and 180 s for hDAT, hNET and rGAT1 by removing the tritiated substrate and washing the cells with 200  $\mu$ L of ice-cold KHB. Afterwards, the cells were lysed in 100  $\mu$ L 1% sodium dodecyl sulfate (SDS) per well. Uptake of tritiated substrate was determined with a beta-scintillation counter (Perkin Elmer, Waltham, MA, USA). In the 4,4'-DMAR assays, non-specific uptake was assessed in presence of 10  $\mu$ M paroxetine for SERT, 10  $\mu$ M mazindole for hDAT, 10  $\mu$ M nisoxetine for NET and 10  $\mu$ M tiagabine for rGAT1. For the MDMA assays, non-specific uptake was assessed in presence of 100  $\mu$ M paroxetine for SERT, 30  $\mu$ M cocaine for hDAT and 1000  $\mu$ M cocaine/methylenedioxypyrovaleron (MDPV) for NET. Non-specific uptake was subtracted from the data to yield specific uptake values. Uptake in the absence of test drugs was defined as 100% and uptake in the presence of drugs was expressed as a percentage thereof. The half maximal inhibitory concentration was determined by non-linear regression fits according to the equation:  $[Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(X - \text{LogEC}_{50}) * \text{Hillslope}})]$  (Mayer et al., 2016a). rGAT1 expressing HEK293 cells were used as a negative control because amphetamines and amphetamine-type substances display no activity at GAT1 at pharmacologically relevant concentrations (Seidel et al., 2005).

Uptake into permeabilized PC12 cells attached to PDL-coated 96-well plates was measured as described earlier (Nakanishi et al., 1995), with minor modifications. PC12 cells were pre-incubated with 150  $\mu$ M digitonin for 15 min at room temperature to permeabilize cell membranes. Subsequently, the cells were washed with HTMS buffer (50 mM Hepes-Tris buffer, 6 mM MgCl<sub>2</sub>, 0.32 M sucrose, 2 mM ATP, pH 7.4 – adjusted with MgOH<sub>2</sub>) (Nakanishi et al., 1995). Hundred nM nisoxetine and 100 nM mazindole were added to the buffer to inhibit DAT and NET. The cells were exposed to increasing concentrations of 4,4'-DMAR, MDMA or reserpine, diluted in HTMS buffer, at a final volume of 200  $\mu$ L per well for 5 min. Subsequently, tritiated substrate (0.1  $\mu$ M [<sup>3</sup>H]5-HT) was added. Uptake was terminated after 15 min by aspirating the tritiated substrate and washing the cells with 200  $\mu$ L ice-cold HTMS buffer. Afterwards, the cells were lysed with 100  $\mu$ L 1% SDS per well. This solution was then transferred into counting vials, containing 2 mL scintillation cocktail. Uptake was determined with a beta-scintillation counter (Perkin Elmer, Waltham, MA, USA). Non-specific uptake was determined in presence of 1  $\mu$ M reserpine and subtracted from the data to yield specific uptake values. Uptake in the absence of test drugs was defined as 100% and uptake in the presence of drugs was expressed as a percentage of control uptake. The half maximal inhibitory concentration was determined as mentioned above.

To evaluate VMAT2 uptake inhibition in human striatal tissue, seven samples were derived from autopsied frozen half brains. The specimens originated from voluntary body donations (6 females, 1 male, aged 87  $\pm$  8 years) to the Center of Anatomy and Cell Biology, Medical University of Vienna, and were required to show no evidence of any neurological or psychiatric disorder in their records,

similar to a previous publication (Piffl et al., 2015). 600 mg of striatal tissue were then homogenized in ice-cold 0.3 M sucrose containing 25 mM Tris (pH 7.4) and 10  $\mu$ M pargyline in a glass Teflon Potter-type homogenizer. Vesicles in the supernatants of P2-pellets of a synaptosomal preparation and in H<sub>2</sub>O-lysates of P2-pellets were combined as described recently (Piffl et al., 2014) and stored at –80 °C until uptake analysis. 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  $\mu$ M of [<sup>3</sup>H]dopamine and various concentrations of DMAR, at 30 °C for 4 min as recently described (Piffl et al., 2014).

#### 2.4. Transporter release assays

Our dynamic transporter release assays allow for the assessment of monoamine-transporter-mediated reverse transport and avoid reuptake or retrograde diffusion of tritiated substrates by using a constant flow rate that causes the clearance of released substances (Piffl et al., 1995; Steinkellner et al., 2016; Mayer et al., 2016a). In brief, transporter-expressing cells are grown on glass-coverslips and pre-loaded with tritiated substrate by exposing the cells to 0.4  $\mu$ M [<sup>3</sup>H]5-HT (hSERT), 0.1  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup> (hDAT), 0.05  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup> (hNET) or 0.1  $\mu$ M [<sup>3</sup>H]GABA (rGAT1), respectively, for 20 min at 37 °C. Subsequently, the cells are transferred into small chambers and superfused with KHB (0.7 mL min<sup>-1</sup>). The superfusates are collected in counting vials (10 mL), containing 2 mL scintillation cocktail. After a cycle of 2 min, the next set of vials is automatically filled. The tubes delivering the drug-containing buffer are submerged in a water bath with a constant temperature of 25 °C. To establish a stable basal release, the cells were superfused for 40 min before the collection of 2-min fractions was initiated. At first, three basal fractions were collected before the cells were exposed to monensin (10  $\mu$ M) or solvent for four fractions. The Na<sup>+</sup>/H<sup>+</sup> ionophore monensin was chosen because it disrupts the pre-existing sodium gradient. The examined transporters all belong to the neurotransmitter-sodium-symporter SLC family (NSS). Thus, a dissipated sodium gradient selectively augments efflux triggered by substrates (Sitte and Freissmuth, 2015). Afterwards, the cells were exposed to 10  $\mu$ M 4,4'-DMAR or to a control substance, known to act as transportable substrate of the respective transporter, for five fractions. 3  $\mu$ M *para*-chloramphetamine (PCA) was used for hSERT-expressing cells, 10  $\mu$ M amphetamine for hDAT and hNET and 100  $\mu$ M GABA for rGAT1-expressing cells. rGAT1 expressing HEK293 cells were used as a negative control because amphetamines and amphetamine-type substances display no activity at GAT1 at pharmacologically relevant concentrations (Seidel et al., 2005). Finally, the cells were lysed with 1% SDS. Afterwards the amount of tritiated substrate present each vial was determined by a beta-scintillation counter (Perkin Elmer, Waltham, MA, USA). Efflux of tritium was expressed as a fractional rate, i.e. the radioactivity released during a fraction was expressed as the percentage of the total radioactivity present in the cells at the beginning of that fraction.

#### 2.5. Receptor and transporter binding and activation assays

Receptor and transporter binding affinities were determined as described earlier in detail for each receptor and transporter (Luethi et al., 2017a). In brief, membrane preparations overexpressing the respective receptors or transporters (human genes, with the exception of rat and mouse genes for TAAR1) were incubated with radiolabeled selective ligands at concentrations equal to *K<sub>d</sub>*, and ligand displacement by the compounds was measured. Specific binding was determined as the difference between total binding (binding buffer alone) and nonspecific binding (in the presence of

specific competitors). The radioligands and competitors utilized to determine nonspecific binding are summarized in Table 1.

The compounds were diluted in binding assay buffer (50 mM Tris/HCl, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, pH 7.4). The concentrations tested ranged from 30 pM–30 μM. Membrane suspension radioligand and test compounds were added to the microplates (Greiner, 96-well) at a final volume of 200 μL per well, incubated and shaken for 30 min at room temperature. The binding reaction was terminated by rapid filtration, using Unifilter-96 plates (Packard Instrument Company, PerkinElmer, Schwerzenbach, Switzerland) and pre-soaked GF/C glass filters (incubated for 1 h in 0.3% polyethylenimine, washed with ice-cold washing buffer [50 mM Tris/HCl, pH 7.4]). Afterwards, scintillation cocktail (45 μL/well) was added and the plates were sealed. One hour later, radioactivity was determined by a Microplate Scintillation counter (Packard Instrument Company).

FLIPR assays were conducted as previously described (Luethi et al., 2017a). In brief, HEK293 cells that expressed the human 5-HT<sub>2B</sub> receptor were incubated in PDL-coated 96-well plates overnight. The growth medium was then removed by snap inversion, and 100 μL of calcium indicator Fluo-4 solution (Molecular Probes, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31 °C, the Fluo-4 solution was removed by snap inversion, and 100 μL of Fluo-4 solution was added a second time for 45 min at 31 °C. The cells were then washed and 100 μL assay buffer was added. Thereafter, the plates were placed in a fluorescence imaging plate reader (FLIPR), and 25 μL of the test substances diluted in assay buffer was added online. The increase in fluorescence was measured, and EC<sub>50</sub> values were derived from the concentration-response curves using nonlinear regression. IC<sub>50</sub> values were calculated by use of nonlinear regression curves for one-site models. K<sub>i</sub> values were determined via the Cheng-Prusoff equation ( $K_i = IC_{50}/[1 + \{\text{radioligand concentration}/K_d\}]$ ). The experiments were conducted as concentration-response curves covering 10 individual concentrations for at least three times ( $n = 3-4$ ).

## 2.6. Data and statistical analysis

IC<sub>50</sub>, EC<sub>50</sub> and AUC values were calculated and plotted with Microsoft Excel® 2010 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 7.03 (GraphPad Software Inc., San Diego, CA, U.S.A.), respectively. Transporter ratios were calculated as (1/numerator IC<sub>50</sub>) divided by (1/denominator IC<sub>50</sub>), e.g. the DAT/SERT ratio is expressed as (1/DAT IC<sub>50</sub>) divided by (1/SERT IC<sub>50</sub>) with higher values indicating greater selectivity for DAT. Release of preloaded tritiated substrate in the presence or absence of monensin was analyzed by repeated measures two-way ANOVA (treatment x time) and Šidák's test. All results are expressed as mean ± SEM. *P* values less than 0.05 were considered significant.

## 3. Results

### 3.1. 4,4'-DMAR inhibits transporter-mediated uptake in HEK293 cells

Uptake inhibition experiments were conducted to test whether 4,4'-DMAR interacts with human monoamine transporters (i.e., hSERT, hDAT, hNET) and rGAT1, expressed in HEK293 cells. As shown in Fig. 1, 4,4'-DMAR is a fully efficacious inhibitor of uptake mediated by hDAT, hNET and hSERT. No inhibitory effect could be observed in rGAT1-expressing cells. 4,4'-DMAR inhibited hSERT, hDAT and hNET with equal potency with IC<sub>50</sub> values in the low micromolar range (hSERT = 1.75 μM–95% CI: 1.446 to 2.126; hDAT = 1.04 μM–95% CI: 0.848 to 1.282; hNET = 0.50 μM–95% CI: 0.447 to 0.553). Calculated ratios emphasize 4,4'-DMAR's low selectivity for one transporter over another (DAT/SERT ratio: 1.68; NET/SERT ratio: 3.50; DAT/NET ratio: 0.48). In contrast, even the highest concentration of 4,4'-DMAR tested (1000 μM) failed to achieve half-maximal inhibition of rGAT1-mediated uptake. As a reference comparator compound, we made use of MDMA because of its similar profile of action. MDMA inhibited hSERT (IC<sub>50</sub> = 16.95 μM–95% CI: 13.41 to 21.43), hDAT (IC<sub>50</sub> = 17.62 μM–95% CI: 13.91 to 22.31) and hNET (IC<sub>50</sub> = 4.57 μM–95% CI: 2.93 to 7.28). We did not conduct uptake inhibition experiments in rGAT1-expressing cells because we have, in the same cell line, shown before that MDMA does not interact with this transporter (Rosenauer et al., 2013).

### 3.2. 4,4'-DMAR induces transporter-mediated release in HEK293 cells

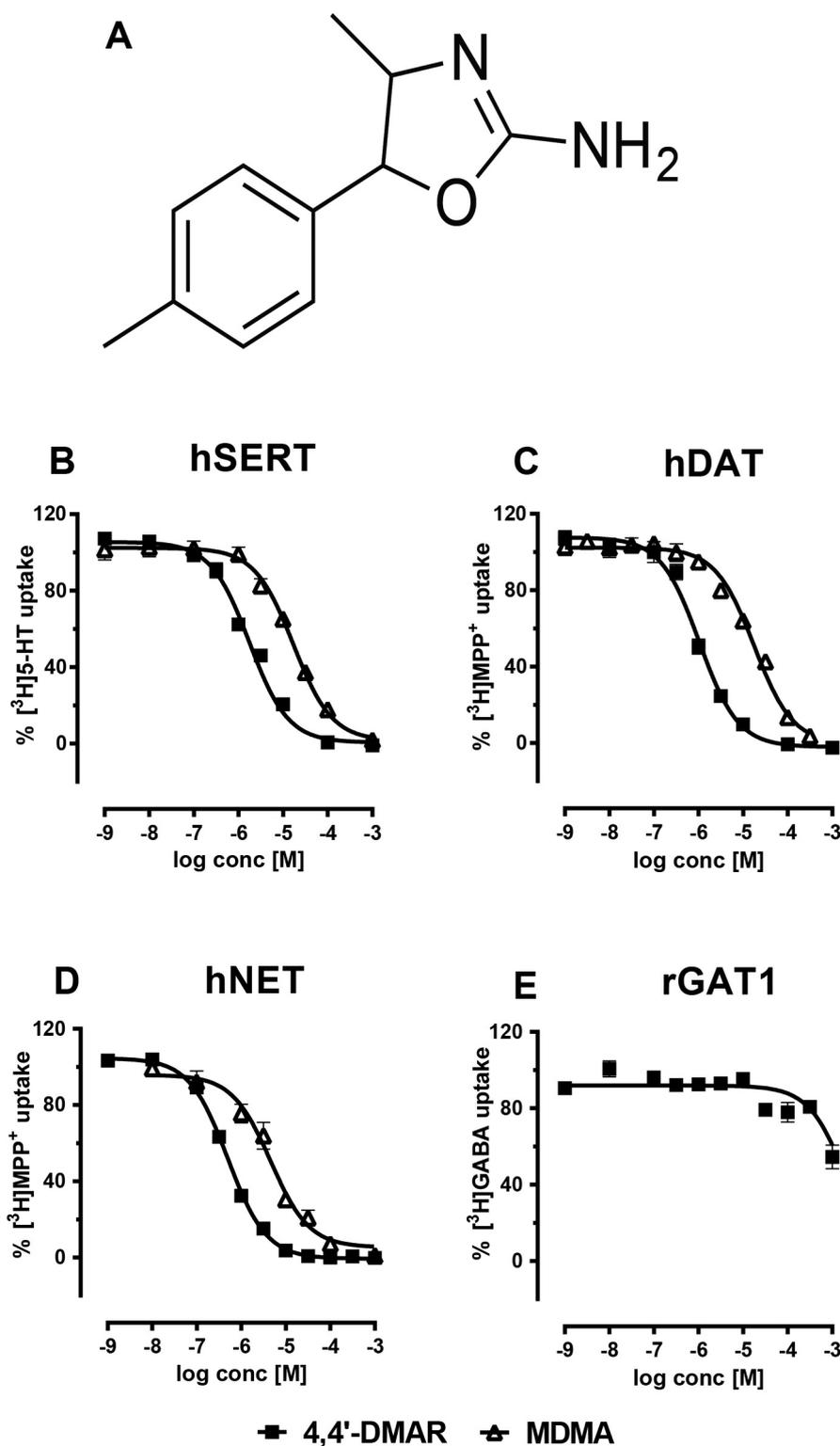
Data gained from uptake inhibition assays alone are unable to reveal whether a drug acts as an inhibitor or as a substrate of transporters (Scholze et al., 2000; Mayer et al., 2016b). Hence, we performed release assays to investigate whether 4,4'-DMAR induces transporter-mediated reverse transport. Dynamic superfusion experiments provide a decisive tool to monitor the effects of test drugs on plasmalemmal transporters (Piffl et al., 1995; Scholze et al., 2000). Cells expressing the transporter of interest were pre-loaded with radiolabeled substrate and exposed to 4,4'-DMAR (10 μM) in the absence or presence of monensin (10 μM). Monensin is a Na<sup>+</sup>/H<sup>+</sup> ionophore, aiding the influx of sodium into the cytosol and dissipating the sodium gradient (Scholze et al., 2000). Thus, monensin selectively augments substrate-induced release. In contrast, the effects of non-transported inhibitors remain unchanged (Mayer et al., 2016a; Scholze et al., 2000).

As shown in Fig. 2, the presence of 4,4'-DMAR drastically augmented the basal release of preloaded [<sup>3</sup>H]substrate via hDAT, hNET and hSERT. On the contrary, 4,4'-DMAR had no effect on the release of [<sup>3</sup>H]GABA from rGAT1 expressing cells. Two-way ANOVA (monensin treatment x time) revealed that monensin treatment

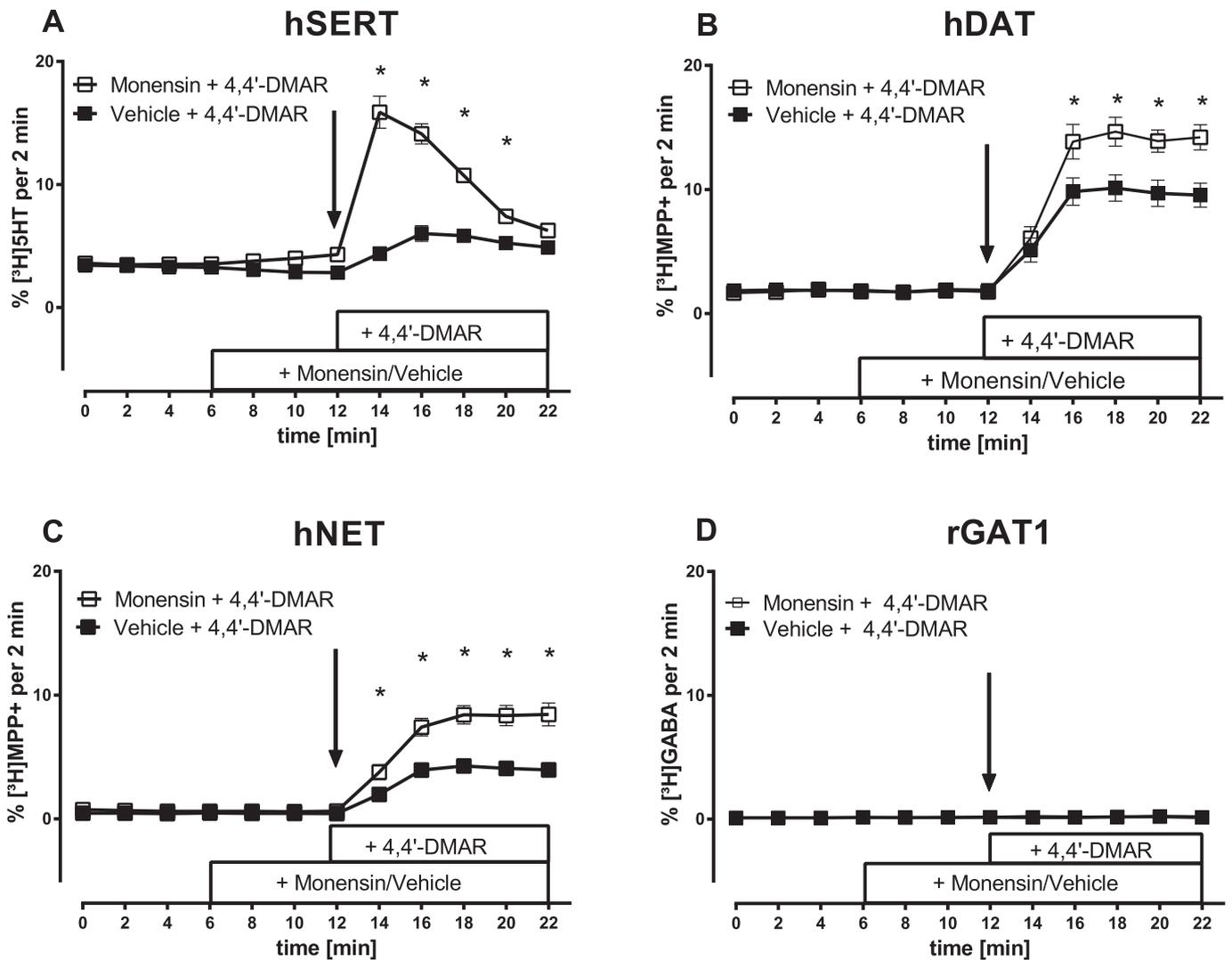
**Table 1**

Receptors and transporters with their respective radioligands and non-specific binding determining substances, as used for radioligand binding assays.

| Receptor             | rTAAR1                     | mTAAR1                       | 5-HT <sub>1A</sub>          | 5-HT <sub>2A</sub>          | 5-HT <sub>2C</sub>                   |                             |
|----------------------|----------------------------|------------------------------|-----------------------------|-----------------------------|--------------------------------------|-----------------------------|
| Radioligand          | [ <sup>3</sup> H]RO5166017 | [ <sup>3</sup> H]RO5166017   | [ <sup>3</sup> H]-8-OH-DPAT | [ <sup>3</sup> H]Ketanserin | [ <sup>3</sup> H] Mesulergine        |                             |
| Concentration        | 3.5 nM                     | 2.4 nM                       | 0.9 nM                      | 0.4 nM                      | 1.4 nM                               |                             |
| Non-specific binding | 10 μM RO5166017            | 10 μM RO5166017              | 10 μM pindolol              | 10 μM spiperone             | 10 μM mianserin                      |                             |
| K <sub>d</sub>       | 2.8 nM                     | 2.0 nM                       | 1.39 nM                     | 0.45 nM                     | 1.6 nM                               |                             |
| Receptor/transporter | α <sub>1A</sub>            | α <sub>2A</sub>              | D <sub>2</sub>              | hDAT                        | hNET                                 | hSERT                       |
| Radioligand          | [ <sup>3</sup> H]Prazosin  | [ <sup>3</sup> H]Rauwolscine | [ <sup>3</sup> H]Spiperone  | [ <sup>3</sup> H]WIN35,428  | N-methyl-[ <sup>3</sup> H]Nisoxetine | [ <sup>3</sup> H]Citalopram |
| Concentration        | 0.106 nM                   | 2.0 nM                       | 1.16 nM                     | 3.3 nM                      | 2.9 nM                               | 1.5 nM                      |
| Non-specific binding | 10 μM chlorpromazine       | 10 μM phentolamine           | 10 μM spiperone             | 10 μM indatraline           | 10 μM indatraline                    | 10 μM indatraline           |
| K <sub>d</sub>       | 0.044 nM                   | 2.0 nM                       | 0.26 nM                     | 30 nM                       | 37 nM                                | 20 nM                       |



**Fig. 1.** Effects of ( $\pm$ )-*cis*-4,4'-dimethylaminorex (4,4'-DMAR) and 3,4-methylenedioxyamphetamin (MDMA) on transporter-mediated uptake in HEK293 cells expressing hSERT, hDAT, hNET and rGAT1, respectively. (A) The chemical structure of 4,4'-DMAR. (B–E) Uptake of the indicated tritiated substrate into cells expressing the indicated transporters was determined in the presence of increasing concentrations of 4,4'-DMAR and MDMA. All symbols represent mean values  $\pm$  SEM. The following numbers indicate the number of individual experiments with 4,4'-DMAR, performed in triplicate: hSERT: 5; hDAT: 5; hNET: 8; rGAT1: 8. The following numbers indicate the number of individual experiments with MDMA, performed in triplicate or duplicate: hSERT: 5; hDAT: 4; hNET: 6.



**Fig. 2.** Effects of 4,4'-DMAR on transporter-mediated release of preloaded radiolabeled substrate from HEK293 cells expressing hSERT, hDAT, hNET and rGAT1, respectively. (A–D) Effects of 4,4'-DMAR on transporter-mediated efflux of tritiated substrates in HEK293 cells expressing monoamine transporters. After three basal fractions monensin or vehicle was added at  $t = 6$  min (MON,  $10 \mu\text{M}$ , indicated by black bar). Subsequently, the cells were exposed to 4,4'-DMAR at  $t = 12$  min (indicated by arrow and bar). All data are represented as mean  $\pm$  SEM. Data were analyzed by repeated measures two-way ANOVA followed by Šidák's test. \* Denotes  $p < 0.05$  when compared to vehicle. The following numbers indicate the number of individual experiments performed in triplicate: hSERT: 5; hDAT: 5; hNET: 5; rGAT1: 5.

significantly influenced the fractional release of [ $^3\text{H}$ ]substrate in presence of 4,4'-DMAR in DAT, NET and SERT expressing cells. The effect of treatment at hDAT was  $F_{1,26} = 4.25$ ,  $P < 0.05$ , for hNET it was  $F_{1,25} = 24.7$ ,  $P < 0.05$  and for hSERT it was  $F_{1,28} = 373.22$ ,  $P < 0.05$ . On the other hand, the results at rGAT1 ( $F_{1,24} = 0.3$ ,  $P = 0.59$ ) revealed that monensin did not affect the effects of 4,4'-DMAR on rGAT1. For a comparison with control substances see Fig. 5 in the appendix.

### 3.3. 4,4'-DMAR binds to monoamine transporters

The binding affinities (and activation potency at  $5\text{HT}_{2\text{B}}$ ) of 4,4'-DMAR are listed in Table 2. 4,4'-DMAR did not bind to rTAAR1, mTAAR1,  $5\text{HT}_{1\text{A}}$ ,  $\alpha_{1\text{A}}$ ,  $\alpha_{2\text{A}}$  and  $\text{D}_2$  at the tested concentrations. 4,4'-DMAR bound to  $5\text{-HT}_{2\text{A}}$  and  $5\text{-HT}_{2\text{C}}$  at higher concentrations with  $K_i$  values of  $8.8 \pm 0.9 \mu\text{M}$  and  $11.1 \pm 0.6 \mu\text{M}$  respectively. As shown in Table 2, 4,4'-DMAR binds to the monoamine transporters hDAT, hNET and hSERT with significantly higher binding affinities when compared to monoamine

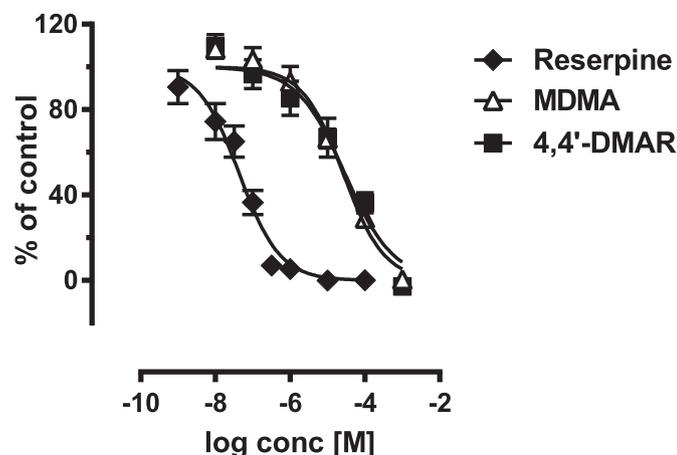
receptors. One-way ANOVA (Tukey's multiple comparisons test) revealed that the  $K_i$  difference between  $5\text{HT}_{2\text{A}}$  and  $5\text{HT}_{2\text{C}}$  and the monoamine transporters is significant ( $p < 0.0001$ ) but the differences between the transporters'  $K_i$  values are only significant when hNET and hSERT are compared ( $p < 0.05$  [a comparison of hDAT and hSERT yields a  $p$  value of 0,053]).

### 3.4. 4,4'-DMAR inhibits VMAT2 uptake in rat PC12 cells

To investigate whether 4,4'-DMAR inhibits rVMAT2, we performed uptake inhibition assays in PC12 cells that endogenously express rVMAT2 on monoaminergic vesicles. We found that reserpine, MDMA and 4,4'-DMAR inhibited rVMAT2 in a concentration-dependent manner (Fig. 3). Reserpine inhibited rVMAT2 with an  $\text{IC}_{50}$  value of  $0.043 \mu\text{M}$  (95% CI:  $0.028 \mu\text{M}$ – $0.067 \mu\text{M}$ ). MDMA and 4,4'-DMAR were much weaker in that regard, with  $\text{IC}_{50}$  values of  $26.47 \mu\text{M}$  (95% CI: 17.45 to 39.9) and  $29.28 \mu\text{M}$  (95% CI: 16.29 to 52.61), respectively.

**Table 2**  
Receptor and transporter binding affinities and 5-HT<sub>2B</sub> activation potencies of (as determined by FLIPR assay) of 4,4'-DMAR.  $K_i$  and  $EC_{50}$  values are given in nM (mean  $\pm$  SD).

| Receptor             | rTAAR1                     | mTAAR1                     | 5-HT <sub>1A</sub>           | 5-HT <sub>2A</sub>                    | 5-HT <sub>2B</sub>                  | 5-HT <sub>2C</sub>                      |
|----------------------|----------------------------|----------------------------|------------------------------|---------------------------------------|-------------------------------------|---|
|                      | Receptor binding           | Receptor binding           | Receptor binding             | Receptor binding                      | Activation potency                  | Receptor binding                        |
|                      | $K_i \pm$ SD [nM]<br>>5010 | $K_i \pm$ SD [nM]<br>>4740 | $K_i \pm$ SD [nM]<br>>17,400 | $K_i \pm$ SD [nM]<br>8846 $\pm$ 862.6 | $EC_{50} \pm$ SD [nM]<br>>10,000    | $K_i \pm$ SD [nM]<br>11,068 $\pm$ 561.1 |
| Receptor/Transporter | $\alpha_{1A}$              | $\alpha_{2A}$              | D <sub>2</sub>               | hDAT                                  | hNET                                | hSERT                                   |
|                      | Receptor binding           | Receptor binding           | Receptor binding             | Transporter binding                   | Transporter binding                 | Transporter binding                     |
|                      | $K_i \pm$ SD [nM]<br>>2120 | $K_i \pm$ SD [nM]<br>>4970 | $K_i \pm$ SD [nM]<br>>13,500 | $K_i \pm$ SD [nM]<br>533.8 $\pm$ 44.2 | $K_i \pm$ SD [nM]<br>266.8 $\pm$ 57 | $K_i \pm$ SD [nM]<br>1881 $\pm$ 183.1   |



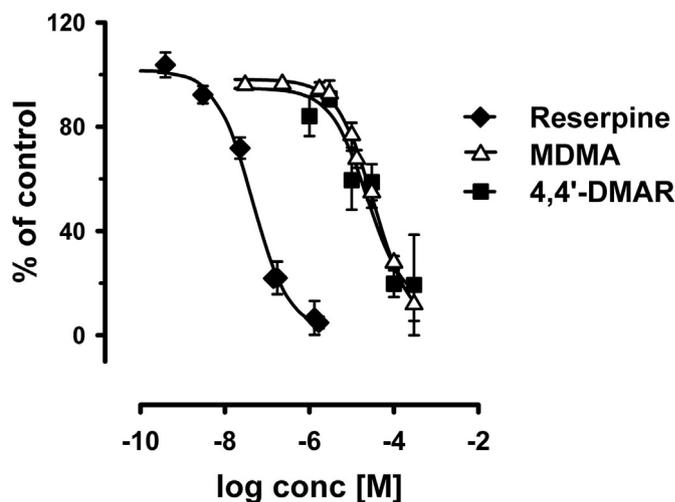
**Fig. 3.** Effects of 4,4'-DMAR, MDMA and reserpine on rVMAT2-mediated uptake in PC12 cells. Uptake inhibition experiments were performed as described in section 2.3. All symbols represent mean values  $\pm$  SEM. The following numbers indicate the number of individual experiments performed in triplicate: 4,4'-DMAR: 8; MDMA: 8; reserpine: 5.

### 3.5. 4,4'-DMAR inhibits VMAT2-mediated uptake in human striatal synaptic vesicles

Finally, we conducted uptake inhibition experiments in synaptic vesicles prepared from human striatum. The experiments were performed in sodium-free potassium phosphate buffer (Pifl et al., 2014). Fig. 4 shows that 4,4'-DMAR inhibits [<sup>3</sup>H]dopamine uptake by human VMAT2 in the micromolar range ( $IC_{50}$ =27.6  $\pm$  7.7  $\mu$ M), comparable to MDMA ( $IC_{50}$ =28.3  $\pm$  4.1  $\mu$ M), but much less potently than reserpine ( $IC_{50}$ =0.044  $\pm$  0.006  $\mu$ M).

## 4. Discussion

The main intention of the present study was to determine the pharmacodynamic effects of ( $\pm$ )-*cis*-4,4'-dimethylaminorex on monoamine receptors and transporters since only two studies are available on 4,4'-DMAR (Brandt et al., 2014; McLaughlin et al., 2015). Both studies used rat brain synaptosomes to determine the releasing effects of 4,4'-DMAR at monoamine transporters. Therefore, we set out to gain information on the effects of 4,4'-DMAR on the human isoforms of DAT, NET and SERT, focusing on uptake inhibition and transporter release experiments, as well as determining its interaction with monoamine transporters and receptors in binding assays. Moreover, we included rat and human VMAT2 in our study since perturbations of VMAT2 function appear to be associated with the long-term toxicity of drugs of abuse (German et al., 2015). This study adds important insights to the existing knowledge on the effects of 4,4'-DMAR in humans. So far, knowledge is limited to subjective drug user reports (Loi et al., 2017). By



**Fig. 4.** Effects of 4,4'-DMAR, MDMA and reserpine on VMAT2-mediated uptake in human striatal synaptic vesicles. Uptake was determined as described in section 2.3. Symbols represent mean values  $\pm$  SEM. Control uptake was 18.6  $\pm$  2.7 pmol  $\times$  4 min<sup>-1</sup>  $\times$  mg protein<sup>-1</sup>. The data of each experiment were fitted by nonlinear regression assuming a Hill slope of 1. The following numbers indicate the number of individual experiments performed in duplicate: 4,4'-DMAR: 6; MDMA: 7; reserpine: 4.

corroborating the results garnered from *ex vivo* rat brain studies obtained in synaptosomes, the findings of this study certainly strengthen the translational relevance of rodent models for analyses of the properties of psychostimulant drugs.

Uptake inhibition experiments demonstrated that 4,4'-DMAR inhibited inwardly-directed transport mediated by hNET, hDAT and hSERT with comparable potencies. We have also shown that it is a more potent inhibitor of uptake in monoamine transporters than MDMA. To test whether 4,4'-DMAR acts an inhibitor or as a substrate-type releaser, we investigated the effect of the drug on carrier-mediated reverse transport. Administration of monensin significantly augmented 4,4'-DMAR evoked release at all three monoamine transporters. We have previously shown that the Na<sup>+</sup>/H<sup>+</sup> ionophore monensin only augments release triggered by substrates, but not inhibitors (Mayer et al., 2016a; Steinkellner et al., 2016; Baumann et al., 2013; Scholze et al., 2000). Hence, these data strongly argue against the possibility that the apparent drug-induced release of tritiated substrates simply represents inhibition of uptake, i.e. “unmasking” of basal substrate leakage. This interpretation is supported by the fact that monensin had no influence on an effect of 4,4'-DMAR on rGAT1-expressing cells but markedly enhanced the releasing effect of the physiological rGAT1-substrate GABA. Similarly, McLaughlin et al. (2015), using rat brain synaptosomes, demonstrated that 4,4'-DMAR is an equipotent releaser at DAT, NET, and SERT with higher potencies compared to the non-selective releaser MDMA. Brandt et al. (2014) have compared the

releasing properties of 4,4'-DMAR to 4-MAR and aminorex and found that the potencies at DAT and NET were comparable to aminorex but less potent than 4-MAR. In contrast, the highest potency to induce 5-HT release via SERT was observed with 4,4'-DMAR. Numerous publications show that ring-substitution in the para-position renders substrate-type releasers less selective for DAT over SERT (Mayer et al., 2017; Solis et al., 2017; Bonano et al., 2015; Sakloth et al., 2015; Cozzi et al., 2013). It is striking that the binding/uptake ratio of 4,4'-DMAR is close to unity, which has been recently shown to generally be the case for uptake inhibitors but not substrates (Eshleman et al., 2017). Nevertheless, previous research from our laboratory suggests that drugs acting on monoamine transporters may alter in adopting either substrate-type or inhibitory binding modes (Sandtner et al., 2016). Furthermore, a lack in conformity between uptake inhibition and radioligand binding assays under varied conditions has previously been observed for pure uptake inhibitors (Luethi et al., 2017b). Our results therefore describe a unique profile of the investigated substance 4,4'-DMAR.

Only one death due to 4-methylaminorex (4-MAR) (Davis and Brewster, 1988) and several cases of pulmonary hypertension caused by aminorex and 4-MAR (Rothman et al., 1999; Gaine et al., 2000) have been reported. Rothman et al. (1999) attribute these to the pronounced effect on 5-HT; most likely, this effect could be attributed to 5-HT<sub>2</sub> receptors (Lauder et al., 2000). The admission notes and autopsy reports of 4,4'-DMAR related deaths (EMCDDA, 2015), suggest clinical features consistent with serotonin toxicity (Greenier et al., 2014). Cardiotoxicity appears to be related to the effects on extracellular norepinephrine, as well as 5-HT (Brandt et al., 2014; Baumann and Rothman, 2009; Lauder et al., 2000). Similar effects and mechanisms have been reported for MDMA (Hysek et al. 2011, 2012; Pifl et al., 2005; Liechti and Vollenweider, 2000).

It must be noted that the neurotoxicity of 4-MAR has already been subject of great controversy. On the one hand, long-term damage to serotonergic and dopaminergic axons has been reported (Bunker et al., 1990; Hanson et al., 1992), but could not be reproduced in another study (Zheng et al., 1997).

Perturbation of VMAT2-function has been linked to neurotoxicity. Hence, we sought to examine the effects of 4,4'-DMAR on VMAT2. We used rat PC12 cells, which endogenously express rVMAT2, and vesicles prepared from human striatum. We found that 4,4'-DMAR inhibited the rat and human isoforms of VMAT2. For both species, the potencies were comparable to those of MDMA. Empirical data bolster the hypothesis that inhibition of VMAT2 might be responsible for long-term neurotoxicity (Lohr et al., 2015; Pifl et al., 2015). Furthermore, substrate-activity and transporter-associated currents have been linked to depletion of 5-HT by fenfluramine in serotonergic neurons (Baumann et al., 2014). Hence, one might speculate that dual substrate activity at plasmalemmal and vesicular transporters results in neurotoxicity (Freyberg et al., 2016; Chaudhry et al., 2007). Future studies on NPS should include assays to determine activity at VMAT2 to provide a detailed understanding of the substances' effects on monoamine transporters.

VMAT2 and ABCB1 (P-glycoprotein, MDR1) are co-expressed in endothelial cells lining up the blood-brain barrier. They share sequence homology and cross-reactivity of a number of inhibitors, e.g. reserpine (Staal et al., 2001). Therefore, 4,4'-DMAR-attributed inhibition of VMAT2-mediated uptake hints at the possibility of a potential ABCB1-inhibition (Staal et al., 2001): This can facilitate the entry of drugs through the blood-brain barrier, which finally leads to accumulation in neurons and increased neurotoxic effects (Schinkel, 1999). The recently described increased brain-to-plasma ratio of 4,4'-DMAR in comparison to its parent substances, might

potentially be caused by 4,4'-DMAR's interaction with ABCB1, at least in part (Lucchetti et al., 2017).

The results of this paper are highly relevant. Even though 4,4'-DMAR has been placed under international control, it still appears to be advertised for sale from Internet retailers (e.g., Shenzhen Chemicals, 2018). According to the EMCDDA (2015), 4,4'-DMAR was often consumed unintentionally as an adulterant that was added to MDMA or cocaine, possibly to increase revenues to drug dealers (Brunt et al., 2017; Giné et al., 2014). Similar reports are available for 4-MAR, which has been sold as cocaine or methamphetamine (Meririnne et al., 2004). Experimental findings presented in Brandt et al. (2014), McLaughlin et al. (2015) and the present study suggest that 4,4'-DMAR resembles the pharmacological profile of MDMA and acts as a non-selective monoamine transporter releasing agent which also affects VMAT2. 4,4'-DMAR might also be considered a more serotonergic drug than the closely related substances 4-MAR and aminorex. The *in vitro* study carried out in rat brain synaptosomes reported by McLaughlin et al. (2015) confirmed that 4,4'-DMAR was a more potent releaser than MDMA across all three transporters. The transporter binding experiments results presented in this study indicate that 4,4'-DMAR exhibits higher binding affinities than MDMA (Simmler et al., 2013, 2014).

Receptor-binding experiments suggest that 4,4'-DMAR exhibits no – or if at all only poor-affinity towards mouse and rat TAAR1. On the contrary, sub- (rat) and low-micromolar (mouse) affinities towards TAAR1 have been reported for MDMA (Simmler et al., 2013). The exact role of TAAR1 in amphetamine action remains far from being completely understood (Sitte and Freissmuth, 2015). However, TAAR1 appears to exert auto-inhibitory effects on monoaminergic neurons, thus regulates the release of the corresponding monoamines (Revel et al., 2011, 2012). TAAR1 is activated by a subset of amphetamines (Simmler et al., 2016). This observation has been linked to auto-inhibitory and neuroprotective effects of TAAR1 in amphetamine action (Miner et al., 2017; Revel et al., 2012; DiCara et al., 2011; Lindemann et al., 2008). The lack of agonist-activity at TAAR1 might further contribute to long-term toxicity of 4,4'-DMAR, thus representing an interesting field for future investigations. Some binding was observed at 5HT<sub>2A</sub> and 5HT<sub>2C</sub> receptors. The affinity towards 5HT<sub>2A</sub> ( $K_i = 8.8 \mu\text{M}$ ) is rather low and comparable to the affinity reported for MDMA ( $K_i = 5.9 \mu\text{M}$ ) (Simmler et al., 2013). Interestingly, the 5-HT<sub>2</sub> subgroup of receptors (5-HT<sub>2A</sub>, 5-HT<sub>2B</sub> and 5-HT<sub>2C</sub>), expressed not only in the CNS, but amongst other peripheral regions, also in the myocardium, endocardium and the heart valves, is implicated in cardiovascular complications (Baumann and Rothman, 2009; Lauder et al., 2000). It has been suggested for MDMA that binding to the 5-HT<sub>2B</sub> receptor is associated with an increased occurrence of valvular heart disease (Baumann and Rothman, 2009). Even though 4,4'-DMAR only binds to 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors with lower affinities, this mechanism might be, in part, implicated in long-term cardiac complications.

While there is now accumulating knowledge on the *in vitro* pharmacodynamic effects of 4,4'-DMAR, its pharmacokinetic properties in humans remain to be determined. However, Lucchetti et al. (2017) have recently evaluated the pharmacokinetic properties in male Wistar rats: They located four metabolites, determined that 4,4'-DMAR is lipophilic enough to easily cross the blood-brain barrier. In addition, the substance was shown (i) to increase spontaneous locomotor activity and (ii) to cause positive motivational and addictive effects in rats (Lucchetti et al., 2017). The latter finding can be corroborated by the high releasing potency at hDAT, as confirmed by the current study. In contrast, MDMA's pharmacokinetic properties in humans have been studied in more detail (De la Torre et al., 2004; Kolbrich et al., 2008). Future animal model studies should explore the potential short-term cardiotoxic and

long-term neurotoxic effects of 4,4'-DMAR, discussed in this study, *in vivo*.

The NPS market is exceedingly dynamic. Hence, the information presented herein might be relevant for our understanding for other aminorex derivatives that may be introduced into the drug markets (McLaughlin et al., 2015). Serotonin syndrome and noradrenergic sympathomimetic effects have been associated with the lethal 4,4'-DMAR intoxications. These insights lend support to medical professionals to treat drug users appropriately. Considering our current understanding of the pharmacology of MDMA and 4,4'-DMAR, it appears unlikely that the adverse effects reported for 4,4'-DMAR are attributable to its interaction with a single molecular target. Data available so far rather suggest that the clinically adverse effects result from overdosing, for example, when 4,4'-DMAR has been mistaken with another drug, be it unintentionally or during the process of adulteration. This emphasizes the important role of harm reduction initiatives, such as the Trans European Drug Information (TEDI) project, where drug users can get their purchased substances, anonymously and without the threat of legal repercussions, chemically analyzed and are informed about the ingredients and the toxicity associated therewith (Brunt et al., 2017). In addition, this strategy allows the collection of temporally resolved insights into the drug markets (Brunt et al., 2017). This might aid the prevention of further drug-related tragedies such as the 4,4'-DMAR-caused series of deaths between June 2013 and February 2014 (EMCDDA, 2015).

## 5. Conclusion

The new psychoactive substance 4,4'-DMAR has been shown to be a potent non-selective monoamine transporter releasing agent that inhibits VMAT2-mediated uptake in human and rat cells. The latter result might explain its potential long-term neurotoxicity, caused by the accumulation of substrate in the cytosol. Compared to other amphetamine-type stimulants and its predecessors, 4,4'-DMAR has a very pronounced serotonergic profile of action similar to MDMA. However, 4,4'-DMAR was often mislabeled and sold as ecstasy, albeit it is a more potent releasing agent. The deaths can therefore be apprehended as overdoses, acutely causing serotonin and norepinephrine toxicity.

## Conflicts of interest

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

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## Appendix B. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.neuropharm.2018.06.018>.

## References

- Baumann, M.H., Volkow, N.D., 2016. Abuse of new psychoactive substances: threats and solutions. *Neuropsychopharmacology* 41, 663–665.
- Baumann, M.H., Bulling, S., Benaderet, T.S., Saha, K., Ayestas, M.A., Partilla, J.S., et al., 2014. Evidence for a role of transporter-mediated currents in the depletion of brain serotonin induced by serotonin transporter substrates. *Neuropsychopharmacology* 39, 1355–1365.
- Baumann, M.H., Partilla, J.S., Lehner, K.R., Thorndike, E.B., Hoffman, A.F., Holy, M., et al., 2013. Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive 'bath salts' products. *Neuropsychopharmacology* 38, 552–562.
- Baumann, M.H., Rothman, R.B., 2009. Neural and cardiac toxicities associated with 3,4-methylenedioxymethylamphetamine (MDMA). *Int. Rev. Neurobiol.* 88, 257–296.
- Bonano, J.S., Banks, M.L., Kolanos, R., Sakloth, F., Barnier, M.L., Glennon, R.A., et al., 2015. Quantitative structure–activity relationship analysis of the pharmacology of *para*-substituted methcathinone analogues. *Brit Pharmacol* 172, 2433–2444.
- Brandt, S.D., Baumann, M.H., Partilla, J.S., Kavanagh, P.V., Power, J.D., Talbot, B., et al., 2014. Characterization of a novel and potentially lethal designer drug, ( $\pm$ )-*cis*-*para*-methyl-4-methylaminorex (4,4'-DMAR, or "Serotoni"). *Drug Test. Anal.* 6, 684–695.
- Bunker, C.F., Johnson, M., Gibb, J.W., Bush, L.G., Hanson, G.R., 1990. Neurochemical effects of an acute treatment with 4-methylaminorex: a new stimulant of abuse. *Eur. J. Pharmacol.* 180, 103–111.
- Brunt, T.M., Nagy, C., Bücheli, A., Martins, D., Ugarte, M., Beduwe, C., et al., 2017. Drug testing in Europe: monitoring results of the trans European drug information (TEDI) project. *Drug Test. Anal.* 9, 188–198.
- Chaudhry, F.A., Edwards, R.H., Fonnum, F., 2007. Vesicular neurotransmitter transporters as targets for endogenous and exogenous toxic. *Annu. Rev. Pharmacol. Toxicol.* 48, 277–301.
- Cozzi, N.V., Brandt, S.D., Daley, P.F., Partilla, J.S., Rothman, R.B., Tulzer, A., et al., 2013. Pharmacological examination of trifluoromethyl ring-substituted methcathinone analogs. *Eur. J. Pharmacol.* 699, 180–187.
- Davis, F.T., Brewster, M.E., 1988. A fatality involving U4Euh, a cyclic derivative of phenylpropanolamine. *J. Forensic Sci.* 33, 549–553.
- De la Torre, R., Farré, M., Roset, P.N., Pizarro, N., Abanades, S., Segura, M., et al., 2004. Human pharmacology of MDMA. Pharmacokinetics, metabolism, and disposition. *Ther. Drug Monit.* 26, 137–144.
- DiCara, B., Maggio, R., Aloisi, G., Rivet, J., Lundius, E.G., Yoshitake, T., et al., 2011. Genetic deletion of trace amine 1 receptors reveals their role in auto-inhibiting the actions of ecstasy (MDMA). *J. Neurosci.* 31, 16928–16940.
- EMCDDA, 2015. Risk assessment report of a new psychoactive substance: 4-methyl-5-(4-methylphenyl)-4,5-dihydrooxazol-2-amine: EU European monitoring Centre for drugs and drug addiction. <http://www.emcdda.europa.eu/system/files/publications/1864/TDAK14005ENN.pdf>. (Accessed 19 March 2018).
- Eshleman, A.J., Wolfrum, K.M., Reed, J.F., Kim, S.O., Swanson, T., Johnson, R.A., Janowsky, A., 2017. Structure-activity relationships of substituted cathinones, with transporter binding, uptake and release. *J. Pharmacol. Exp. Therapeut.* 360, 33–47.
- Freyberg, Z., Sonders, M.S., Aguilar, J.L., Hiranita, T., Karam, C.S., Flores, J., et al., 2016. Mechanisms of amphetamine action illuminated through optical monitoring of dopamine synaptic vesicles in *Drosophila* brain. *Nat. Commun.* 7, 10652.
- Gainé, S., Rubin, L., Kmetzo, J., Palevsky, H., Traill, T., 2000. Recreational use of aminorex and pulmonary hypertension. *Chest* 118, 1496–1497.
- German, C.L., Baladi, M.G., McFadden, L.M., Hanson, G.R., Fleckenstein, A.E., 2015. Regulation of the dopamine and vesicular monoamine transporters: pharmacological targets and implications for disease. *Pharmacol. Rev.* 67, 1005–1024.
- Giné, C.V., Espinosa, I.F., Vilamala, M.V., 2014. New psychoactive substances as adulterants of controlled drugs. A worrying phenomenon? *Drug Test. Anal.* 6, 819–824.
- Greenier, E., Lukyanova, V., Reede, L., 2014. Serotonin syndrome: fentanyl and selective serotonin reuptake inhibitor interactions. *AANA J. (Am. Assoc. Nurse Anesth.)* 82, 340–345.
- Hanson, G.R., Bunker, C.F., Johnson, M., Bush, L., Gibb, J.W., 1992. Response of monoaminergic and neuropeptide systems to 4-methylaminorex: a new stimulant of abuse. *Eur. J. Pharmacol.* 218, 287–293.
- Hofmaier, T., Luf, A., Seddik, A., Stockner, T., Holy, M., Freissmuth, M., et al., 2014. Aminorex, a metabolite of the cocaine adulterant levamisole, exerts amphetamine-like actions at monoamine transporters. *Neurochem. Int.* 73, 32–41.
- Hysek, C.M., Simmler, L.D., Nicola, V., Vischer, N., Donzelli, M., Krähenbühl, S., et al., 2012. Duloxetine inhibits effects of MDMA ("ecstasy") *in vitro* and in humans in a randomized placebo-controlled laboratory study. *PLoS One* 7, e36476.
- Hysek, C.M., Simmler, L.D., Ineichen, M., Grouzmann, E., Hoener, M.C., Brenneisen, R., et al., 2011. The norepinephrine transporter inhibitor reboxetine reduces stimulant effects of MDMA ("ecstasy") in humans. *Clin. Pharmacol. Ther.* 90, 246–255.
- Kankaanpää, A., Meririnne, E., Ellermaa, S., Ariniemi, K., Seppälä, T., 2001. Detection and assay of *cis*- and *trans*-isomers of 4-methylaminorex in urine, plasma and tissue samples. *Forensic Sci. Int.* 121, 57–64.
- Kolbrich, E.A., Goodwin, R.S., Gorelick, D.A., Hayes, R.J., Stein, E.A., Huestis, M.A., 2008. Plasma pharmacokinetics of 3,4-methylenedioxymethylamphetamine after controlled oral administration to young adults. *Ther. Drug Monit.* 30, 320–332.
- Lauder, J.M., Wilkie, M.B., Wu, C., Singh, S., 2000. Expression of 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub> and

- 5-HT<sub>2C</sub> receptors in the mouse embryo. *Int. J. Dev. Neurosci.* 18, 653–662.
- Liechti, M.E., Vollenweider, F.X., 2000. The serotonin uptake inhibitor citalopram reduces acute cardiovascular and vegetative effects of 3,4-methylenedioxymethamphetamine ('Ecstasy') in healthy volunteers. *J. Psychopharmacol.* 14, 269–274.
- Lindemann, L., Meyer, C.A., Jeanneau, K., Bradala, A., Ozmen, L., Bluethmann, H., et al., 2008. Trace amine associated receptor 1 modulates dopaminergic activity. *J. Pharmacol. Exp. Therapeut.* 32, 948–956.
- Lohr, K.M., Stout, K.A., Dunn, A.R., Wang, M., Salahpour, A., Guillot, T.S., Miller, G.W., 2015. Increased vesicular monoamine transporter 2 (VMAT2; Slc18a2) protects against methamphetamine toxicity. *ACS Chem. Neurosci.* 6, 790–799.
- Loi, B., Zloh, M., De Luca, M.A., Pintori, N., Corkery, J., Schifano, F., 2017. 4,4'-Dimethylaminorex ("4,4'-DMAR"; "Serotoni") misuse: a web-based study. *Hum. Psychopharmacol. Clin. Exp.* 32, e2575.
- Lucchetti, J., Marzo, C.M., Di Clemente, A., Cervo, L., Gobbi, M., 2017. A validated, sensitive HPLC-MS/MS method for quantification of cis-para-methyl-4-methylaminorex (cis-4,4'-DMAR) in rat and human plasma: application to pharmacokinetic studies in rats. *Drug Test. Anal.* 9, 870–879.
- Lueithi, D., Trachsel, D., Hoener, M.C., Liechti, M.E., 2017a. Monoamine receptor interaction profiles of 4-thio-substituted phenethylamines (2C-T drugs). *Neuropharmacology*. <http://10.1016/j.neuropharm.2017.07.012> (Epub ahead of print).
- Lueithi, D., Kaeser, P.J., Brandt, S.D., Krähenbühl, S., Hoener, M.C., Liechti, M.E., 2017b. Pharmacological profile of methylphenidate-based designer drugs. *Neuropharmacology*. <https://10.1016/j.neuropharm.2017.08.020> (Epub ahead of print).
- Mayer, F.P., Burchardt, N.V., Decker, A.M., Partilla, J.S., Li, Y., McLaughlin, G., et al., 2017. Fluorinated phenmetrazine "legal highs" act as substrates for high-affinity monoamine transporters of the SLC6 family. *Neuropharmacology*. <https://doi.org/10.1016/j.neuropharm.2017.10.006> (Epub ahead of print).
- Mayer, F.P., Luf, A., Nagy, C., Holy, M., Schmid, R., Freissmuth, M., Sitte, H.H., 2016a. Application of a combined approach to identify new psychoactive street drugs and decipher their mechanisms at monoamine transporters. In: Baumann, M., Glennon, R., Wiley, J. (Eds.), *Neuropharmacology of New Psychoactive Substances (NPS)*, vol. 32. Springer, New York, pp. 333–350. *Current Topics in Behavioral Neurosciences*.
- Mayer, F.P., Wimmer, L., Dillon-Carter, O., Partilla, J.S., Burchardt, N.V., Mihovilovic, M.D., et al., 2016b. Phase 1 metabolites of mephedrone display biological activity as substrates at monoamine transporters. *Br. J. Pharmacol.* 173, 2657–2668.
- McLaughlin, G., Morris, N., Kavanagh, P., Power, J., Twamley, B., O'Brien, J., et al., 2015. Synthesis, characterization, and monoamine transporter activity of the new psychoactive substance 3',4'-methylenedioxy-4-methylaminorex (MDMAR). *Drug Test. Anal.* 7, 555–564.
- Meririnne, E., Ellermaa, S., Kankaanpää, A., Bardy, A., Seppälä, T., 2004. Pharmacokinetics and tissue distribution of the stereoisomers of 4-methylaminorex in the rat. *J. Pharmacol. Exp. Therapeut.* 309, 1198–1205.
- Miner, N.B., Elmore, J.S., Baumann, M.H., Phillips, T.J., Janowsky, A., 2017. Trace amine-associated receptor 1 regulation of methamphetamine-induced neurotoxicity. *Neurotoxicology* 63, 57–69.
- Nakanishi, N., Onozawa, S., Matsumoto, R., Hasegawa, H., Minami, N., 1995. Vesicular monoamine uptake by digitonin-permeabilized PC12 Cells: inhibitory effect of neuromodulators and drugs on the amine transport. *J. Biochem.* 118, 291–296.
- Piffl, C., Nagy, G., Berényi, S., Kattinger, A., Reither, H., Antus, S., 2005. Pharmacological characterization of ecstasy synthesis byproducts with recombinant human monoamine transporters. *J. Pharmacol. Exp. Therapeut.* 314, 346–354.
- Piffl, 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.
- Piffl, C., Rajput, A., Reither, H., Blesa, J., Cavada, C., Obeso, J.A., et al., 2014. Is Parkinson's disease a vesicular dopamine storage disorder? Evidence from a study in isolated synaptic vesicles of human and nonhuman primate striatum. *J. Neurosci.* 24, 8210–8218.
- Piffl, C., Drobny, H., Reither, H., Hornykiewicz, O., Singer, E.A., 1995. Mechanism of the dopamine-releasing actions of amphetamine and: plasmalemmal dopamine transporter versus vesicular monoamine. *Mol. Pharmacol.* 47, 365–373.
- Poos, G.L., Carson, J.R., Rosenau, J.D., Roszkowski, A.P., Kelley, N.M., McGowin, J., 1963. 2-Amino-5-aryl-2-oxazolines. Potent new anorectic agents. *J. Med. Chem.* 6, 266–272.
- Revel, F.G., Moreau, J., Gainetdinov, R.R., Bradaia, A., Sotnikova, T.D., Mory, R., et al., 2011. TAAR1 activation modulates monoaminergic neurotransmission, preventing hyperdopaminergic and hypoglutamatergic activity. *Proc. Natl. Acad. Sci. Unit. States Am.* 108, 8485–8490.
- Revel, F.G., Moreau, J., Gainetdinov, R.R., Ferragud, A., Velázquez-Sánchez, C., Sotnikova, T.D., et al., 2012. Trace amine associated receptor 1 partial agonism reveals novel paradigm for neuropsychiatric therapeutics. *Biol. Psychiatr.* 72, 934–942.
- Rosenauer, R., Luf, A., Holy, M., Freissmuth, M., Schmid, R., Sitte, H.H., 2013. A combined approach using transporter-flux assays and mass spectrometry to examine psychostimulant street drugs of unknown content. *ACS Chem. Neurosci.* 4, 182–190.
- Rothman, R.B., Ayestas, M.A., Dersch, C.M., Baumann, M.H., 1999. Aminorex, fenfluramine, and chlorphentermine are serotonin transporter substrates. Implications for primary pulmonary hypertension. *Circulation* 100, 869–875.
- Sakloth, F., Kolanos, R., Mosier, P.D., Bonano, J.S., Banks, M.L., Partilla, J.S., et al., 2015. Steric parameters, molecular modelling and hydrophobic interaction analysis of the pharmacology of para-substituted methcathinone analogues. *Br. J. Pharmacol.* 172, 2210–2218.
- Sandtner, W., Stockner, T., Hasenhuettel, P.S., Partilla, J.S., Seddik, A., Zhang, Y.W., et al., 2016. Binding mode selection determines the action of ecstasy homologs at monoamine transporters. *Mol. Pharmacol.* 89, 165–175.
- Schinkel, A.H., 1999. P-Glycoprotein, a gatekeeper in the blood–brain barrier. *Adv. Drug Deliv. Rev.* 36, 179–194.
- Scholze, P., Zwach, J., Kattinger, A., Piffl, 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.
- Seidel, S., Singer, E.A., Just, H., Farhan, H., Scholze, P., Kudlacek, O., et al., 2005. Amphetamines take two to tango: an oligomer-based counter-transport model of neurotransmitter transport explores the amphetamine action. *Mol. Pharmacol.* 67, 140–151.
- Shenzhen Chemicals, 2018. 4,4'-DMAR. <http://shenzhenchemicals.com/product/44-dimethylaminorex-44-dmar-cas-1445569-01-6/>. (Accessed 18 January 2018).
- Simmler, L.D., Buchy, D., Chaboz, S., Hoener, M.C., Liechti, M.E., 2016. In vitro characterization of psychoactive substances at rat, mouse, and human trace amine-associated receptor 1. *J. Pharmacol. Exp. Therapeut.* 357, 134–144.
- Simmler, L.D., Rickli, A., Hoener, M.C., Liechti, M.E., 2014. Monoamine transporter and receptor interaction profiles of a new series of designer cathinones. *Neuropharmacology* 79, 152–160.
- Simmler, L.D., Buser, T.A., Donzelli, M., Schramm, Y., Dieu, L.H., Huwyler, J., et al., 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.
- Solis, E., Partilla, J.S., Sakloth, F., Ruchala, I., Schwientek, K.L., De Felice, L.J., et al., 2017. N-Alkylated analogs of 4-methylamphetamine (4-MA) differentially affect monoamine transporters and abuse liability. *Neuropsychopharmacology* 42, 1950–1961.
- Staal, R.G.W., Yang, J., Hait, W.N., Sonsalla, P.K., 2001. Interactions of 1-methyl-4-phenylpyridinium and other compounds with P-glycoprotein: relevance to toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Brain Res.* 910, 116–125.
- Steinkellner, T., Mayer, F.P., Holy, M., Montgomery, T., Eisenrath, B., Freissmuth, M., Sitte, H.H., 2016. Tracer measurements to study outward transport by neurotransmitter transporters. In: Bönisch, H., Sitte, H.H. (Eds.), *Neurotransmitter Transporters*. Springer, Berlin, pp. 23–40.
- UNODC, 2016. Commission on Narcotic Drugs decision on international control of PMMA,  $\alpha$ -PVP, 4,4'-DMAR, MXE and Phenazepam enters into force. <https://www.unodc.org/LSS/Announcement/Details/6dd8eae4-7b30-4ae1-889e-f8a03d62df18>. (Accessed 19 March 2018).
- UNODC, 2017. World drug report. <https://www.unodc.org/wdr2017/index.html>. (Accessed 19 March 2018).
- Zheng, Y., Russell, B., Schmierer, D., Laverty, R., 1997. The effects of aminorex and related compounds on brain monoamines and metabolites in CBA mice. *J. Pharm. Pharmacol.* 49, 89–96.